Phenotype and Cytokine Expression Profiles of Ovine Dendritic cells Migrating to Lymph Nodes.

Samantha L. Bailey

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I hereby declare that the work presented in this thesis is my own. Any contributions by others are clearly acknowledged.

Samantha Bailey
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ABSTRACT

Dendritic cells (DCs) function at the interphases of innate and adaptive immunity. The ability of DCs to control the responses of effector T cells, B cells and NK cells are due, in part, to cell contacts and DC cytokines. Different DC subsets exist, that appear to have different biological roles *in vivo*. Cannulation of the 'pseudoafferent' lymphatics is one of the few methods of obtaining differentiated DCs *ex vivo*. Lymph DCs are not a homogenous population, and this study tested the hypothesis that discrete populations exist *in vivo*, that differentially express immunomodulatory molecules. Two lymph DC populations were defined by expression of signal regulatory protein-α (SIRPα). SIRPα$^+$ DCs were CD58$^+$, CD11a$^+$, WC6$^+$, CD45RA$^-$, expressed CD16, CD14, CD206, CD4 and CD8 at low levels or on minor sub-populations, and as a single population expressed higher levels of CD11c, CD40, CD80, CD86 and MHC II than did SIRPα$^-$ DCs. SIRPα$^-$ DCs were CD16$^-$, CD14$^-$, CD206$^-$, CD4$^-$, CD8$^-$, CD11a$^+$ and displayed heterogeneity of expression of CD58, CD11c, WC6 and CD45RA. Importantly, freshly isolated SIRPα$^+$ DCs expressed IL-12 p40 and IL-18 mRNA, whereas SIRPα$^-$ DCs were negative for these transcripts. Neither IL-6 nor IL-10 mRNA were detected by PCR in the DC populations. Differential expression of molecules involved in antigen uptake (CD14, CD16, CD206), interactions with lymphocytes (CD54, CD11a, CD40) and antigen presentation and T cell activation (MHC II, CD80, CD86, IL-12, IL-18), suggests that the lymph DC populations may have different funtions *in vivo*. This knowledge would support therapeutic strategies to target particular DC populations *in vivo*, to enhance, or to suppress their natural role in the immune response.

In order to perform multiple cytokine analysis on the lymph DCs, a multiprobe RNase protection assay (RPA) was developed for eleven cytokines. First, sheep interleukin-18 (IL-18) was cloned and sequenced. By inducing the production of interferon gamma, IL-18 is an important regulator of T cell immunity. This was the first description of IL-18 expression patterns in sheep. Second, standardisation analysis showed that the RPA could reliably quantify levels of IL-1β, IL-4, IL-6,

IL-10, IL-12 p40, IL-18, GM-CSF, IFNγ and TGFβ mRNA. IL-8 and TNFα - specific riboprobes require optimisation for use with the developed protocol. The amount of RNA required for analysis using the RPA exceeded what was isolated from the DC subsets. However, the RPA makes it possible to evaluate levels of nine cytokine mRNA transcripts in single RNA samples isolated from sheep. This will help support studies of ovine immunology and immunopathology, where cytokine responses are the readout.

Abbreviations

aa	Amino acid
AM	Alveolar macrophage
AP	Activator protein
A/F	Ascitic fluid
APC	Antigen presenting cell
ATP	Adenosine triphosphate
ALC	Afferent lymph cell
ALVC	Afferent lymph veiled cell
APS	Ammonium persulphate
BAL	Bronchoaleolar lavage
aBAL	Adherent bronchoalveolar cell
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD40L	CD40 ligand
CCR7	Chemotactic cytokine receptor 7
cDNA	Complementary DNA
CpG DNA	Cytosine-phosphatase-guanine oligodeoxynucleotides
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DDC	Dermal dendritic cell
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucloside triphosphate
dTTP	Deoxythymidine triphosphae
dH_2O	Distilled water
ds	Double stranded
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent activated cell sorting
Fas-L	Fas ligand
FCS	Fetal calf serum
FITC	Florescein isothiocyanate
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphatase-dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
GPI	glycosyl phosphatidyl-inositol
HBSS	Hank's buffered salt solution
HEPES	N'-[2-hdroxyethyl] piperazine-N'-[2-ethanesulphonic acid]

Abbreviations

HEV	High endothelial venules
IC	antigen:antibody immune complex
ICAM	Intracellular adhesion molecule
ICSBP	Interferon-consensus sequence binding protein
IFN	Interferon
Ig	Immunoglobulin
IGIF	Interferon-gamma inducing factor
IL	Interleukin
IL-18BP	IL-18-binding protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAK	Interleukin-1 receptor-associated kinase
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JNK	N-terminal kinase
kb	Kilobase
kDA	Kilodalton
LAD	Leucocyte adhesion deficiency
LB	Luria Bertani
LBP	Lipopolysaccharide-binding protein
LC	Langerhans' cell
LPS	Lipopolysaccharide
Mal	MyD88-adaptor like
MACS	Magnetic cell separation
MAPK	MAP kinase
MCS	Multiple cloning site
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIP1α	Macrophage inflammatory protein-α
MoDC	Monocyte-derived DC
MR	Mannose receptor
mAb	Monoclonal antibody
min	Minutes
mRNA	Messenger RNA
NFκB	Nuclear factor-kappa B
NK	Natural killer
NMS	Normal mouse serum
NRS	Normal rat serum
nt	Nucleotide
$OD_{(x)}$	Optical density at x nanometers
PAGE	Polyacrylamide gel electophoresis
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoethrin
PHA	Phytohaemagglutanin

Abbreviations

PKR	Protein kinase receptor
PMA	Phorobol 12-myristate 13-acetate
PMT	Photo multiplier tube
PRR	Pattern recognition receptor
p	Prefix for plasmid DNA
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPA	RNase protection assay
RPMI	Rosewell Park Memorial Institute
RT-PCR	Reverse transcriptase PCR
rt	Room temperature
rATP	Riboadenosine triphosphate
rCTP	Ribocytidine triphosphate
rGTP	Riboguanosine triphosphate
rNTP	Ribonucloside triphosphate
rTTP	Ribothymidine triphosphate
SDS	Sodium dodecyl sulphate
SHPS-1	SHP substrate-1
SH	Src-homology-domain containing phosphatase
SIRP	Signal regulatory protein
STAT	Signal-transducing activator of transcription
SLC	Secondary lymphoid tissue chemokine
SSC	Side scatter
S/S	Saturated supernate
s	Prefix for sterile
TAE	Tris acetate EDTA buffer
TEMED	N, N, N', N'-tetraethylmethylenedamine
TGF	Transforming growth factor
Th	T helper cell
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOLLIP	Toll-interacting protein
TRAF	TNF receptor-associated factor 6
U	Unit
UTR	Untranslated region
UV	Ultraviolet light
VCAM	Vascular cell adhesion molecule-1
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

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Chapter 1: Introduction

Dendritic cells (DCs) are crucial components of the immune response, owing to their essential role in the induction and control of T cell immunity, and modulating the function of B cells and NK cells. DCs comprise a diverse group of cell subsets with different functions. Functional diversity is related to the DC anatomical location, differentiation state, antigenic stimulus and interactions with effector cells (Banchereau et al., 2000).

Obtaining DCs for analysis is not straightforward, and may explain why DCs are one of the most recently identified leucocyte types (Steinman and Cohn, 1973). DCs comprise less than 2% of cells in peripheral lymphoid organs, and their isolation from these sites requires enzymatic digestion of the collagenase matrix (Steinman, Lustic and Cohn, 1974). DC precursors are resident in haematopoietic tissue and circulate in blood (Caux et al., 1992), and can be propagated *in vitro* in the presence of cytokine growth factors. These techniques likely modulate DC phenotype and function. There are few models where differentiated DC can be obtained *ex vivo* with minimal manipulation. One technique involves the cannulation of the lymphatics and collection of lymph DCs. Leucocytes defending epidermal and mucosal tissues migrate to the lymph nodes via the afferent lymphatics. This project utilised the 'psuedoafferent' lymphatic cannulation model in sheep (Hall, 1966) (Section 2A.2). This model was so named because it involves the ablation of the draining lymph node. Over a period of 4-6 weeks the numerous afferent lymphatics re-anastomose with the single efferent lymphatic. Afferent lymph now flows into the efferent lymphatic that is fitted with a cannula allowing lymph cells to be collected externally. Afferent lymph is the principal pathway for DC and macrophage entry to the lymph nodes (Vestweber, 2003). The advantage of assessing afferent lymph DCs is that they represent these cells *in vivo*. This thesis is primarily a descriptive study of ovine afferent lymph DC populations. Constitutive attributes of lymph DCs were assessed, such as surface phenotype and cytokine expression. An assay for cytokine expression analysis was developed to support the studies of DC function. This Introduction will discuss aspects of DC function and biology.

1.1 DC Populations

1.1.1 Murine DCs

At least four populations of DCs have been described in murine lymphoid organs, which can be separated by expression of CD11c and the CD8αα homodimer (CD8α) (Anjuere et al., 1999; Kamath et al., 2000; Asselin Paturel et al., 2001). Three DC populations in peripheral lymph nodes have been defined by CD8α expression: two of these are $CD8\alpha^+$ and $CD8\alpha^-$ DCs, that have been termed 'lymphoid' and 'myeloid' DCs, respectively (Section 1.2), and are also present in the spleen. The third express intermediate levels of CD8α ($CD8\alpha^{int}$), and derive from epidermal Langerhans cells (LCs) that up-regulate CD8α expression in the lymph node (Anjuere et al., 1999), and unless otherwise stated will be grouped with the $CD8\alpha^+$ DC population. These DCs all express CD11c, have a mature phenotype, and are potent stimulators of T cell activation and proliferation (Kamath et al., 2000). The DC populations however, differ in localisation and function. In the spleen and lymph nodes (LN), $CD8\alpha^+$ DCs are found primarily in the periarteriolar lymphatic sheaths (PALS), that are rich in T cells, whereas $CD8\alpha^-$ DCs are situated at the marginal zones (Pulendran et al., 1997; Leenen et al., 1998). Endotoxin induces $CD8\alpha^-$ DC migration to the PALS where they present processed antigen collected in the periphery (Reis e Sousa and Germain, 1999). $CD8\alpha^+$ DCs produce higher amounts of interleukin- (IL-) -12p70 favouring a T helper- (Th-)1 response, and are less phagocytic than $CD8\alpha^-$ DCs (Maldonado-Lopez et al., 1999; Hochrein et al., 2001). Ultimately, DCs function as potent inducers of T cell proliferation and differentiation to support adaptive immunity. The CD8α subsets show considerable plasticity in the type of T cell responses they induce. This is discussed further in Section 1.8.

A fourth murine DC population, identified as $CD11c^{lo}$ $CD8\alpha^+$ with expression of the CD45 isoform B220 are functionally equivalent to plasmacytoid DCs (pDC) described in humans (Asselin Paturel et al., 2001). These DCs will subsequently be termed $B220^+$ pDCs. pDCs have an immature phenotype, are poor stimulators of T cells, and induce the generation of regulatory T cells *in vitro* (Bilsborough et al., 2003). It has therefore been hypothesised by a number of investigators (Martin et al., 2002; Bilsborough et al., 2003), that pDCs function *in*

vivo to support peripheral T cell tolerance (Section 1.7). $B220^+$ pDCs express CD8α at various levels, and it is likely that they are responsible for the attributes of $CD8\alpha^+$ DCs ascribed with tolerogenic / suppressive function (Steinman et al., 2000). pDCs are the major interferon (IFN) -α producers *in vivo* during infection with virus, and rapidly mature and produce IL-12 following stimulation with cytosine-phosphatase-guanine oligodeoxynucleotides (CpG DNA), that mimic bacterial DNA sequences (Asselin Paturel et al., 2001).

1.1.2 Human DCs

The majority of studies of human DCs are performed on cells derived from precursors in umbilical cord blood ($CD34^+$) (Romani et al., 1996) or from $CD14^+$ blood monocytes cultured with cytokines such as granulocyte monocyte colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNFα) (Caux et al., 1992) and IL-4. There is a lack of CD8α expression on human DCs (Banchereau et al., 2000), so direct analogies with mouse DC subsets are not plausible. $CD14^+$ monocytes cultured in the presence of GM-CSF and IL-4 give rise to DCs, termed DC1, that produce high levels of IL-12 after activation, and preferentially induce the generation of Th1 cells (Sallusto and Lanzavecchia, 1994; Romani et al., 1996). DC1 thus resemble murine $CD8\alpha^+$ DCs. pDCs were termed DC2 because of a propensity to induce Th2 differentiation that is unaffected by IL-4 or IL-12 (Rissoan et al., 1999). Similar to murine DC subsets, human DC1 and DC2 have subsequently been found to exhibit plasticity of responses, which probably reflects more closely the biology of DCs *in vivo* (Section 1.8).

1.1.3 Cutaneous DCs

DCs are most numerous in tissues underlying body surfaces (skin, trachea, gut). Represented in the epidermis by LCs, skin DCs act as sentinels and sequester antigen for presentation to T cells in the draining lymph nodes (Inaba et al., 1986; Larsen et al., 1990; Romani et al., 1989; Weinlich et al., 1998; Ruedl et al., 2000), and LCs are particularly potent at inducing T cell proliferation in primary mixed leukocyte reactions (MLRs) (Schuler and Steinman, 1985; Inaba et al. 1986). To enable efficient DC:lymphocyte interactions, peripheral DCs are required to travel to

secondary lympoid tissues. The lymphatic cannulation model in sheep demonstrated that DCs undergo steady-state migration to lymph nodes via afferent lymph draining the skin (Hopkins et al., 1985). DC migration is discussed further in Section 1.5. Afferent lymph DCs are largely comprised of LCs (Brand et al., 1999; Hoefsmit, Duijvestijn and Kamperdijk, 1982). Moreover, a number of studies indicated that mature LCs comprise a significant proportion of DCs in murine LNs. These studies utilised fluorescent labels in skin sensitisers (Anjuere et al., 1999; Kamath et al., 2000; Salomon et al., 1998; Ruedl et al., 2000); BrdU uptake *in vivo* (Kamath et al., 2000); and specific LC phenotypic markers, E-cadherin and Birbeck granules (Ruedl et al., 2000). The mature form of LCs have the slowest turnover rate of the LN DC populations, with a half-life of around 15 days (Salomon et al., 1998; Kamath et al., 2000; Ruedl et al., 2000). This increases the chance of interacting with rare antigen specific T cells, and mounting a specific adaptive response against antigens collected in the skin. Mechanisms of DC / LC antigen uptake and presentation are discussed in more detail in Section 1.3.

DCs situated in the dermis (dermal DC; DDC) have an immature phenotype and weak T cell stimulatory potential (Nestle et al., 1993). In the LN, the mature form of DDCs have been proposed to be a $CD8\alpha^-$ DC population with immature phenotype, that turn over with faster kinetics than LCs (Kamath et al., 2000). Given that the mature forms of both LCs and DDCs are found in significant numbers in cutaneous-draining lymph nodes (Kamath et al., 2000), it is likely they both contribute to immunological protection of the skin.

1.2 DC Ontogeny

DCs originate from bone marrow (BM) precursors (Katz, Tamaki and Sachs, 1979; Steinman, 1991; Inaba et al., 1993). Haematopoietic reconstitution studies in mice, and *in vitro* studies using murine and human DCs, suggest a plasticity of both myeloid and lymphoid pathways that lead to the generation of various DC subtypes.

1.2.1 A Myeloid Lineage for DCs

Early studies demonstrated a common myeloid precursor cell for most DCs. Inaba et. al. (1993) demonstrated that DCs, monocyte / macrophages and

granulocytes can be generated from haematopoietic precursors when cultured with GM-CSF. In addition, *in vitro*-derived $CD14^+$ precursor cells (Caux et al., 1996) or $CD14^+$ blood monocytes (Sallusto and Lanzavecchia, 1994) differentiate into DCs. This has also been demonstrated *in vivo* (Randolph et al., 1999).

1.2.2 A Lymphoid Lineage for DCs

The identification of murine $CD4^{lo}$ thymic precursors that generated DCs, T and B lymphocytes and NK cells demonstrated a lymphoid-related lineage for DCs (Wu et al., 1991). Transfer of these precursors into irradiated recipients led to the development of T lymphocytes and a subset of $CD8\alpha^+$ DCs that lacked myeloid markers in the spleen, thymus and lymph nodes (Ardavin et al., 1993).

1.2.3 Common DC Precursors

However, the concept of different DC lineages and functions has subsequently been challenged by several findings. Injection of MHC II^- common myeloid precursors (CMPs) into irradiated recipients has shown that they give rise to both $CD8\alpha^+$ and $CD8\alpha^-$ DCs (Traver et al., 2000). Moreover, common lymphoid precursors (CLPs) produce $CD8\alpha^+$ and $CD8\alpha^-$ DCs following *in vivo* transfer (Manz et al., 2001). Martin and colleagues (2002) have also demonstrated a common precursor for $CD8\alpha^+$ and $CD8\alpha^-$ DCs. These workers cited the use of negative selection for the removal of contaminating cells as an explanation as to why the common precursors had not been identified in earlier studies. The experimental data suggests that both myeloid- and lymphoid-lineage multi-potential precursors can give rise to $CD8\alpha^+$ and $CD8\alpha^-$ DCs *in vivo*, and that these cells types are not lineage restricted. Whether the mature DC populations are exclusive, or represent differentiation stages of each other is debatable. Using *in vivo* reconstitution, Manz et al. (2001) concluded that CMPs and CLPs contribute equally to thymic DCs, whereas peripheral DCs were in the most part derived from CMPs. This is supported as CMP:DC precursors express fms-related tyrosine kinase 3 (Flt3) (D Amico and Wu, 2003). Flt3 is a potent growth factor for DCs *in vivo* and *in vitro* (Pulendran et al., 1997; McKenna et al., 2000), and is required for DC development in mice (Brasel et al., 2000). A common DC precursor demonstrates the plasticity of DCs during

development. Accumulating experimental evidence indicates that the environment the cell finds itself has a strong effect on DC biology (Section 1.8). Although they no longer denote separate lineages, the terms of 'lymphoid' and 'myeloid' DCs are still widely used

The development of $B220^+$ pDCs differs from that of other DCs *in vitro*. $B220^+$ pDCs do not require GM-CSF in culture and depend on IL-3, this contrasts with the requirement of *in vitro* derived human (Romani et al., 1994) and murine (Sallusto and Lanzavecchia, 1994) $CD8\alpha^-$ DCs. However, reconstitution experiments demonstrate that $B220^+$ pDCs derive from the same blood (del Hoyo et al., 2002) or haematopoietic $Flt3^+$ precursor (D Amico and Wu, 2003) as both $CD8\alpha^+$ and $CD8\alpha^-$ DCs. Therefore, it seem that $B220^+$ pDCs, like other DC populations may be generated from both CMPs and CLPs.

A separate developmental pathway, however, has been described for epidermal LCs. Bone-marrow chimera experiments demonstrated host LCs 18 months following the transfer of syngeneic, congenic haematopoietic cells (Merad et al., 2002). It seemed that LCs derive from a local precursor that populates the skin under steady state conditions (Merad et al., 2002), which may be the $CD14^+$ DDC (Larregina et al., 2001). MHC II^- precursors were previously identified to give rise to LCs or DCs depending on the cytokines they were cultured with (Caux et al., 1996). Therefore, although the bone marrow precursor may be the same as other DC populations *in vivo*, the developmental pathway of LCs does appear different from other DC populations.

1.3 Antigen Capture, Processing and Presentation by DCs

Although DCs comprise multiple subsets, the potency they exhibit in inducing T cell activation depends on their ability to capture, process and present antigenic peptide on MHC molecules to specific T cells. DCs in the periphery are ideally placed to capture antigen and act as the 'sentinels of the immune surveillance sytstem' (Steinman and Inaba, 1989). These cells are situated in essentially all tissues (with the possible exception of the brain and testes) in the form of immature interstitial DCs and as LCs in the epidermis. Inflammatory stimuli and pathogens or their products induce DC maturation. This, in a co-ordinated manner, regulates the

ability of DCs to capture, process and present antigen, the expression of co-stimulatory and accessory molecules, cytokine production and migration of the DCs to the secondary lymphoid tissue.

Four stages of DC maturational development have been described based on the anatomical location (Austyn, 1996). These include DC progenitors in the BM and blood; immature DCs capable of processing antigen in peripheral non-lymphoid sites; maturing DCs migrating in the afferent lymph and blood; and mature DCs that present antigen in secondary lymphoid tissue. Immature DCs are present in lymphoid and non-lymphoid tissue in the human foetus and newborn rats (Fossum, 1989). DC migration from the BM to peripheral tissue therefore appears to occur in steady state and a pathogen-independent manner.

Immature DCs are very efficient at antigen capture and may use several uptake pathways. These include macropinocytosis, which is constitutive in DCs, and allows continuous uptake of large volumes of fluid that contain particulate antigen (Sallusto et al., 1995). Phagocytosis enables uptake of larger particles such as apoptotic cells (Rubartelli, Poggi and Zocchi, 1997) or virus and bacteria. Finally, receptor-mediated endocytosis via the C-type lectin receptors CD206 and DEC-205 (Jiang et al., 1995; Sallusto et al., 1995; Tan et al., 1997) or Fcγ receptors types I (CD64), II (CD32) and III (CD16), permits uptake of mannosylated antigen and antibody-antigen immune complexes (IC), respectively. Occupation and activation of FcγRs and C-type lectins on DCs results in a number of maturational changes including the modulation of surface molecule expression, and the transcription of cytokines such as IL-12 and TNFα (delaSalle et al., 1997; Lefkowitz et al., 1997; Yamamoto, Klein and Friedman, 1997; Lanzavecchia, 1998).

Once the maturation process of DCs is triggered in the periphery by encounter with pathogen and / or inflammatory cytokines, DCs downregulate expression of endocytic / phagocytic receptors and switch roles from antigen capture to antigen presentation. DCs restrict antigen presentation to those antigens internalised during maturation thus favouring the stimulation of T cells specific for pathogenic agents. Immature DCs accumulate MHC II molecules in lysosome-related intracellular compartments called MHC class II-rich compartments (MIIC), which have a multivesicular and multilamellar structure (Kleijmeer et al., 1995;

Nijman et al., 1995). Soluble and particulate antigens captured by DCs are targeted to MIIC (Inaba et al., 1997). In immature DCs, MHC II molecules are rapidly internalised and have a short half-life. Maturation or inflammatory stimuli increase MHC II molecule synthesis and translocation of MHC II-peptide complexes to the cell surface where they have a high-life of days and are situated for T cell interaction and recognition (Cella et al., 1997; Inaba et al., 1997; Inaba et al., 2000). Mature DCs change expression of other molecules such as increased surface expression of adhesion (CD11a, CD11c), co-stimulatory (CD40, CD80 and CD86) molecules, and the production of cytokines such as IL-12 are also enhanced (Macatonia et al., 1995; Cella et al., 1996; Koch et al., 1996; Reis e Sousa et al., 1997).

1.4 Cross-presentation of Antigen

DCs are capable of shunting cell-associated antigen from endocytic compartments to the MHC class I processing pathway, and presenting that antigen to $CD8^+$ T cells (Heath and Carbone, 2001). This function is termed 'cross-presentation'. Cross presentation is particularly important for the initiation of immune responses against tumours and viruses that do not have access to the classical pathways for MHC class I presentation by antigen presenting cells (APCs). $CD8\alpha^+$ DCs are primarily responsible for cross-presenting antigen associated with captured apoptotic cells *in vivo* (den Haan, Lehar and Bevan, 2000). Fc receptors (FcRs) on the cell surface, are particularly efficient at shuttling antigens to the MHC I processing pathway (Regnault et al., 1999), and den Haan and Bevan (2002) demonstrated that $CD8\alpha^-$ DC can also cross-present exogenous peptide (ovalbumin) in an immune complex / FcR –dependant manner. FcRs are widely expressed on DCs (Regnault et al., 1998) and enable $CD8\alpha^-$ DCs, that are not constitutively capable of cross-presenting antigen, to do so in the event of a humoral protective response in response to pathogens.

1.5 DC Migration

Transport of antigen from the periphery to the regional lymph node for presentation of T cells depends on DC migration. Generally, DCs enter peripheral

lymphoid tissue via the afferent lymphatics that drain peripheral tissues. Studies of epidermal LCs have shown that various mediators such as LPS (Banchereau and Steinman, 1998), heat shock proteins (HSPs) (Binder et al., 2000) and CpG DNA (Ban et al., 2000), as well as the cytokines IL-1β and TNFα promote LC maturation and migration. Molecular events identified during the migration of LCs from the epidermis include down-regulation of E-cadherin, allowing detachment from keratinocytes (Schwarzenberger and Udey, 1996), the up-regulation and activation of adhesion molecules, induction of metalloproteinases which breakdown the extracellular matrix (Kobayashi et al., 1999), and the expression of the chemotactic cytokine receptor 7 (CCR7). The ligands for CCR7 (secondary lymphoid tissue chemokines [SLC] and macrophage inflammatory protein-3α [MIP-3α]) are found in lymphatic endothelium and in secondary lymphoid tissue and are fundamental in the initial stages of LC translocation to the regional lymph nodes (Saeki et al., 1999). The migratory function of DCs is tightly regulated by sequential modulation of chemokines and chemokine receptors (Sallusto et al., 1998; Sallusto et al., 1999).

Once present in the lymph node, integrins expressed on the DCs interact with intercellular adhesion molecule (ICAM) molecules on T cells, promoting adhesion and stimulation, and lowering the concentration of antigen required for stimulation of T cells. DCs receive additional maturation signals from T cells via CD40 ligand (CD40L). DCs attract and cluster with naïve or resting T cells in an antigen-independent manner in order to select rare MHC-peptide-specific T lymphocytes which may be present at a frequency of <1/100 000 (Banchereau and Steinman, 1998).

Using the 'psuedoafferent' lymphatic cannulation model, Hopkins and colleagues (1985) demonstrated that DCs undergo steady-state migration to lymph nodes via afferent lymph draining the skin, but are completely absent in efferent lymph; indicating that DCs terminate in the lymph nodes and die there. Moreover, DCs have been directly observed disappearing from the lymph nodes 24-48 hours following interactions with T cells (Ingulli et al., 1997). Clearing of DCs may be contributed to by MHC II cross-linking- induced apoptosis, whereas, CD40-cognate interactions protect antigen-bearing DC from apoptosis (McLellan et al., 2000), thus retaining DCs during antigen presentation to specific T cells.

1.6 Direct and Indirect Mechanisms of Pathogen Activation of DCs

During the initial stages of a primary infection, DCs constitute an important part of the innate protective response which comprises both immune and non-immune cells. Innate recognition of pathogens is thought to be mediated by pathogen recognition receptors (PRRs) that mediate cell activation (Medzhitov and Janeway, 1997). An important and well-described class of innate receptors are the Toll-like receptors (TLRs), which are members of the IL-1 receptor / TLR superfamily (O'Neill and Greene, 1998). Pathogenic interactions of TLRs on DCs often induce DC maturation (Schnare et al., 2001) (Sections 1.3 and 1.6) and in this manner, DCs function as a link between innate and adaptive immunity. It has been proposed that in addition to carrying antigen for MHC-restricted presentation to T cells (signal 1) and providing co-stimulatory signals (B7 family molecules, signal 2), DC migration from the periphery also carries a third signal (Kalinski et al., 1999). This so-called "signal 3" can be mediated by soluble molecules including IL-12 (Trinchieri, 1998), IL-18 (Micallef et al., 1996), and IFNα (Sareneva, et al., 1998), and polarises the type of immune response generated by the naïve T cell residing in the secondary lymphoid tissue. Different TLR ligands have been shown to induce different cytokine responses in DC. In this manner, the type of pathogen mediates the type of T cell response that develops.

Eleven TLRs have so far been identified (Zhang et al., 2004). Their identified ligands are shown in Table 1. Different TLRs share intracellular signalling pathways and ligation and activation of TLRs triggers DC maturation involving two signalling pathways: ERK kinase, promoting DC survival; and NFκB promoting increased expression of co-stimulatory molecules and pro-inflammatory cytokines (IL-1, TNFα, IL-12) (Rescigno et al., 1998). Interaction and activation of TLRs with different PAMPs or through endogenous mediators (see below) affects DC maturation and the subsequent signals for T cell development. The TLR3 and TLR4 agonists dsRNA and LPS, respectively, trigger DC maturation to a phenotype that produce high levels of bioactive IL-12 p70 and strongly supports the development of Th1 cells (Cella et al., 1999; Pulendran et al., 2001; de Jong et al., 2002). Similarly, TLR7 activation by imiquimod (a compound with potent anti-viral and anti-tumour

properties) (Hemmi et al., 2002), or TLR9 activation by CpG DNA (Hemmi et al., 2002) induces immature CD11c$^+$ DC to support the development of Th1 cells from naïve T cells, mediated by IL-12, or in the case of pDC, IFNα (Krug et al., 2001; Ito et al., 2001). However, monocyte-derived DCs primed with TLR2 stimuli do not promote strong Th1 responses, and produce low levels of IL-12 upon stimulation (Re and Strominger, 2001). TLR2 forms heterodimers with TLR6 during recognition of mycoplasma derived lipopeptides, proteoglycans and the yeast cell wall component zymosan (Underhill et al. 1999). In comparison with LPS primed monocyte DCs, mycoplasma-derived lipopeptide 2 does not induce IL-12 production, but IL-10 (Weigt et al., 2003), and induces unpolarised T cell responses (Qi, Denning and Soong; 2003). Similarly, proteoglycans induce relatively low levels of IL-12 production and high levels of IL-10 (Re and Strominger, 2001). These data imply that TLRs may mediate distinct cytokine profiles in DCs with different consequences for T cell activation.

Toll-like receptor (TLR)	Identified Ligand
TLR2	Bacterial lipoproteins Proteoglycans Yeast zymosan
TL3	double stranded RNA
TLR4	LPS
TLR7	Imiquimod
TLR9	CpG DNA

Table 1 Toll-like receptors and their ligands

Differential expression of TLR by human DC populations confers responsiveness or hyporesponsiveness to the corresponding ligands. For example, TLR9 is expressed by pDCs but not CD11c$^+$ or monocyte-derived DCs. pDCs do not express most other TLR that are expressed by CD11c$^+$ or monocyte-derived DCs, such as the LPS receptor TLR4 (Bauer et al., 2001; Kadowaki et al., 2001; Jarrossay et al., 2001). Accordingly pDCs produce large amounts of IL-12 in response to CpG DNA, and do not respond to LPS; whereas CD11c$^+$ DCs respond strongly to LPS and not CpG DNA (Kadowaki et al., 2001). This is one situation where the type of DC determines the response outcome (Section 1.8).

During infection DCs not only become activated directly by pathogens and PAMPs, but indirectly by endogenous factors of inflammation and tissue damage, signalling 'danger' to the immune system (Matzinger, 1998). These include pro- or anti-inflammatory cytokines, chemokines, heat shock proteins (HSPs), eicosanoids, intercellular lipids associated with necrotic cells, and extracellular matrix breakdown components. Although controversy still exists, evidence is accumulating that these danger-associated endogenous ligands activate PRRs (Beg, 2002), examples include mammalian HSPs inducing intracellular signalling and cytokine secretion by TLR-dependant mechanisms (Basu et al., 2000; Ohashi et al. 2000; Vabulas 2002). The ability of different PAMPs and endogenous mediators to polarise DC responses and the subsequent T cell response may well yet be defined.

1.7 DCs and Peripheral Tolerance

A body of data suggests that DCs may also support peripheral tolerance. DCs efficiently present endogenous antigen in context with MHC II in secondary lymphoid organs (Inaba et al., 1997). Importantly, DCs loaded with apoptotic bodies migrate under steady state conditions from the gut to mesenteric lymph nodes (Huang et al., 2000). In addition, DCs have been visualised taking up apoptotic cells *in vivo* before migration to the T cell areas of the spleen (Morelli et al., 2003). These observations suggest that tissue-resident DCs take up apoptotic cells, process endogenous antigens contained within them and constitutively migrate via the afferent lymphatics to the lymph nodes. In the absence of direct or indirect activation by pathogens (Section 1.6), DCs are thought to support tolerance to self-antigen by inducing anergy / deletion of self reactive T cells or through the generation of T-regulatory (T-reg) cells (Leenen et al., 1998; Dhodapkar et al., 2001; Hawiger et al., 2001).

1.8 DC Plasticity

Early studies on the biological roles of human DC populations indicated that different populations had different roles *in vivo*. Mature DC1, when activated *in vitro* with CD40 ligand (CD40L), produced large amounts of IL-12 and

preferentially induced Th1 development. CD40L-activated DC2, when cultured with IL-3, produced lower amounts of IL-12 and preferentially induced Th2 development (Rissoan et al., 1999; Ito et al., 2001). Furthermore, activated pDC2 produced IFNα and primed naïve T cells to produce IFNγ (Grouard et al., 1997; Olweus et al., 1997; Siegal et al., 1999; Cella et al., 2000).

The opposite to these functions was observed in murine DCs using a different model of DC antigen presentation. Pulendran et al. (2001) used DCs loaded *in vitro* with an ovalbumin (OVA) peptide injected into syngeneic mice reconstituted with OVA-specific T cells. The OVA peptide-pulsed adoptively transferred $CD8\alpha^+$ subset induced high levels of IFNγ, and the $CD8\alpha^-$ subset induced IL-4 and IL-10, as well as IFNγ and IL-2. Selective expansion of these subsets *in vivo* resulted in an increase in antigen-specific titres with different isotypes (Pulendran et al., 2001). Maldonado Lopez et al. (1999) used murine DCs pulsed *ex vivo* with keyhole limpet haemocyanin and showed that different DC subsets directed the development of distinct Th cells. The hypothesis arose that in the mouse $CD8\alpha^+$ lymphoid DCs promote Th1 responses, and $CD8\alpha^-$ myeloid DCs preferentially induce Th2 immune responses.

Certain difficulties arose with this hypothesis of DC subset - immune response determination. The nature of the immune response should depend on the nature of the pathogen (Section 1.6), abundance of the pathogen and tissue microenvironment, and not the sub-population of DC that has captured and presented that antigen. High antigen doses induce myeloid DCs and $B220^+$ pDCs to induce Th1 differentiation, whereas Th2 cells are generated with low antigen doses (Boonstra et al., 2003). IFNγ enhances a Th1-promoting capacity in both $CD8\alpha^+$ and $CD8\alpha^-$ DCs (Maldonado Lopez et al., 2001). DCs from the respiratory tract (Stumbles et al., 1998), Peyer's patches (Iwasaki and Kelsall, 2001), and liver (Khanna et al., 1998) preferentially induce a Th2 response, whereas splenic $CD11c^+$ DCs preferentially induce a Th1 response. This difference may be explained by tissue-specific cytokine microenvironments and exposure to particular pathogens.

In humans, multiple factors have been found to influence DC effector function, including maturation status and signal, DC:T cell ratio and duration of DC activation. Mature DC1 induce a Th1 response and a strong CTL response (Rissoan

et al., 1999), while immature DC1 induce $CD4^+$ and $CD8^+$ T cells which produce IL-10 (Jonuleit et al., 2000; Dhodapkar et al., 2001; Gilliet and Liu, 2002). Immature DC1 stimulated with *Staphylococcus aureus*, CpG, dsRNA, or the T cell signals CD40L and IFNγ induce a Th1 response (Kalinski et al., 1999). However, immature DC1 stimulated in the presence of IL-10, transforming growth factor-β (TGFβ) or steroids induce a Th2 response (Kalinski et al., 1999). pDC2 cultured with IL-3 promote a Th2 response, while pDC2 activated by virus prime naive T cells to produce IFNγ and IL-10 (Kadowaki et al., 2001). A high DC:T cell ratio induces DC1 to promote Th1 responses, whereas low ratios promote a Th2 response (Tanaka et al., 2000). DC produce large amounts of IL-12 and induce a Th1 response during the early stages of activation, but DC that become "exhausted" after prolonged activation no longer produce IL-12 and induce a Th2 response (Langenkamp et al., 2000).

These experiments show that the capacity of DCs to skew the immune response is dependant on the antigen, length of antigen exposure, subtype of DC, tissue of residence, and extent of T lymphocyte interaction. The net result of pathogenic onslaught is a DC system that stimulates, and also regulates the ensuing immune response.

1.8.1 'Suppressor' cell activity

$CD8\alpha^+$ splenic DCs have been demonstrated to be particularly efficient at inducing antigen-specific tolerance *in vivo* (Liu et al., 2002; Legge et al., 2002), and it is $CD8\alpha^+$ DCs in pancreatic islets that maintain local T cell tolerance to endogenous antigen (Belz et al., 2002). Fas-dependant apoptosis is one mechanism of deleting self-reactive T cells in the periphery (Ferguson et al., 2002), and it is the $CD8\alpha^+$ DC subset that mediates Fas-induced apoptosis (Suss and Shortman, 1996). Immature DCs loaded with peptide induce T cell proliferation, but no Th cytokine profiles (Dhodapkar et al., 2001). In addition, DCs that have taken up apoptotic bodies (Albert et al., 1998) or antigen in the presence of IL-10 (Corinti et al., 2001) retain an immature phenotype with low expression of CD80 and CD86. Repetitive stimulation of $CD4^+$ T cells with peptide-loaded immature DCs induces T cell anergy (Jonuleit et al., 2000). It appears that the phenotype associated with suppressor DC

function implies a plasticity, and is not the function of a particular population. This is elegantly illustrated for $B220^+$ pDCs from mouse lymph nodes, which have an immature phenotype when analysed *ex vivo* (Anjuere et al., 1999) and are potent inducers of T cell tolerance *in vitro* (Martin et al., 2002). If, however, $B220^+$ pDCs are matured with CpG DNA, they acquire potent Th1-inducing activity (Boonstra et al., 2003). Different DC subsets have demonstrated plasticity for activation / suppression of T cell responses *in vitro*. However the function of *ex vivo* DCs appears more stringent, and *in vivo* functions remain to be fully elucidated.

1.9 Ovine Lymph DC

The 'psuedoafferent' lymphatic cannulation model system has allowed investigators unique insight into the function of the immune system in associated tissues (Haig, Hopkins and Miller, 1999). Afferent lymph contains lymphocytes (primarily $CD4^+$ memory T cells) and DCs (Hall, 1966; Hopkins et al., 1989; Bujdoso et al., 1989) involved in the immune surveillance of the tissues. Dendritic cells in afferent lymph (lymph DCs) are a mixed population from various sources migrating to draining lymph nodes. Lymph DCs carry antigen from the tissues from which they originate. They are generally equipped for interactions with lymphocytes, and induce responses specific to the antigens they carry.

A role for lymph DCs transporting antigen to lymph nodes for presentation of T cells was demonstrated by Budjoso et al. (1989). Sheep were immunised intradermally at the lymphatic cannulation site with OVA and purified protein derivative of tuberculin (PPD). At different time points following antigen administration, lymph DCs were collected and added to T cell lines specific for OVA or PPD. DCs isolated after OVA immunisation could only stimulate the proliferation of OVA-specific T cells, and DCs isolated after PPD immunisation could only activate PPD-specific T cells. This experiment demonstrated that antigen in the skin is taken up and transported by DCs via the afferent lymphatics to be used to activate T cells in an antigen-specific manner. A mechanism for antigen uptake by sheep lymph DCs was demonstrated by Harkiss et al. (1990) using *in vitro* and *in vivo* methods. They showed that freshly isolated lymph DCs constitutively take up protein antigen at low levels. However, antigen uptake was markedly enhanced in

the presence of IgG_1 or IgG_2 antibodies *in vitro* and *in vivo*, but not $F(ab)_2$ fragments, suggesting that uptake was mediated via FcRs. FcγR engagement induced a reduction of expression of MHC II, CD1, CD44, very late antigen (VLA)-4, leucocyte functional antigen (LFA)-1 and LFA-3 after 8 hours, but an increase of these molecules after 24 hours (Coughlan et al. 1996b). The modulation of MHC molecules and adhesion molecules may concordantly function to maximise presentation of antigen in ICs captured *in vivo* for presentation to T cells in the LN. Further studies demonstrated that T cell stimulatory function was enhanced up to 169-fold when DCs were incubated with antigen in the presence of specific antibody (Couglan et al. 1996a). Presentation of antigen:Ig complexes were mediated via MHC II, demonstrated by the ability of MHCII-specific antibody to block T cell stimulation in these experiments (Couglan et al. 1996a).

Sheep lymph DCs express high levels of MHC I and MHC II as a homogenous population (Budjoso et al., 1990), and levels of MHC II are increased 1 – 2 days following secondary antigen challenge (Hopkins et al., 1989). These experiments demonstrated that lymph DCs change phenotype and function in response to antigen *in vivo*. Using immune complex binding and CD1 expression, Harkiss et al. (1990) demonstrated the existence of four discrete lymph DC populations. Demonstrating heterogeneity of lymph DCs and a complexity of lymph DC populations, which may reflect intrinsic functions of these cells.

1.10 Summary and Project Aims

DCs orchestrate the immune response in both a stimulatory and tolerogenic manner. Discrete DC populations migrate via the lymphatics draining cutaneous tissue in sheep (Harkiss et al., 1990). Studies on the function of particular DC subsets have been conflicting depending on the tissue of residence, DC:lymphocyte ratio, and type and preparation of antigen. Isolating DCs, and analysing their biological characteristics *ex vivo*, minimises the modulation of DC biology during experimental manipulation. Determining the molecular characteristics of DCs provides insights into the biological functions of the cells. For example, DCs with a mature phenotype (MHC II^{hi}, $CD86^+$ $CD40^+$) are extremely efficient at driving naïve T cell proliferation, whereas immature DCs (MHC II^{lo}, $CD86^-$, $CD40^-$) are poor stimulators of T cells (Banchereau and Steinman, 1998). Also, DC IL-10 and IL-6

support Th2 responses (Rincon et al. 1997), whereas IL-18 promotes Th1 development and NK cell activation (Okamura et al. 1998). Determining the *ex vivo* characteristics of sheep lymph DCs may provide insight of the function of these cells *in vivo.*

Project Hypothesis: Subpopulations of sheep lymph DCs differ in function. Functional differences will be evident at the molecular level, such as surface molecule expression and expression of immune modulatory cytokines.

Aim one: Phenotypically characterise sheep lymph DCs. This was achieved using specific antibodies and two-colour flow cytometry. The classes of molecules evaluated included: phenotypic / lineage-associated markers (CD11c, CD4, CD8, CD45RA, WC6 antigen); receptors used for antigen sampling and uptake (CD14, CD16, CD206); molecules involved in interactions with T cells (CD54, CD11a, CD40, signal regulatory protein-α [SIRPα]); antigen presentation (MHC II); and co-stimulation (CD80, CD86). DC expression of SIRPα in cattle (previously termed MyD-1) identifies two distinct populations (Howard et al., 1997). Whether SIRPα expression on sheep DCs is relevant to the determination of individual sub-populations was also addressed in this project.

Aim two: Determine constitutive cytokine expression by sheep lymph DCs. A multiprobe RNase protection assay (RPA) was developed to measure eleven ovine cytokines (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-18, IFNγ, GM-CSF, TGFβ, TNFα) and two housekeeping genes (ATPase, GAPDH). The ovine RPA enabled the anlaysis of cytokines involved in innate inflammatory responses (IL-1β, TNFα, IL-8, GM-CSF), and adaptive immune responses (IL-4, IL-6, IL-10, IL-12p40, IL-18, IFNγ, TGFβ). The cytokines included in the RPA have pleitrophic affects on the immune system, both in action, and the responding cell type. In addition to supporting studies in the current project, the ovine cytokine RPA could be further utilised in a wide array of cytokine studies of ruminants.

Chapter 2: Materials and Methods

The companies supplying the reagents used in this work are referred to in the text. All company addresses are listed in Appendix 2. Cell cultures were carried out at 37°C in 5% carbon dioxide. Fetal calf serum (FCS) (Invitrogen LT) used in the culture media was heat inactivated (56°C, 30 min). All cell culture techniques were carried out in an aseptic manner inside microbiological (class II) safety cabinets using sterile equipment and solutions. Oligonucleotide primers were synthesized by MWG biotech.

2.1 Animals

Male and female Blackface x Finnish Landrace sheep of 1-2 years of age obtained from Edinburgh University Veterinary Faculty were used for the collection of blood, lymph and lung washes.

2A Preparation of Cells

2A.1 Alveolar Macrophages (AM)

Alveolar macrophages (AM) were used for optimising many experimental techniques as these belong to the myeloid cell lineage, and large numbers are relatively easy to obtain using the bronchoalveolar lavage (BAL) technique. Based on the protocol described by Mayer and Lam (1984) lungs, heart and intact trachea were completely removed, and the outer surface washed with H_2O. The bronchoalveolar cells were washed out of the lung using ice-cold sterile (s) Hank's buffered salt solution (HBSS) in 3-4 washes (1.5-2 litres) while the lungs were gently massaged. Alternatively, euthanised animals were intubated with 9.5 – 10.5 mm cuffed endotracheal tubes, and the lung lumen flushed with ice-cold sHBSS while massaging the thoracic cavity. BAL cells were transferred by pipette into 250 ml flasks (Beckman) in order to separate the cells from floating mucus and cell debris from the top of the cell suspension, and then centrifuged at 800 x g for 15 – 25 min at 4°C. Cells were washed twice in sHBSS and recovered at 300 x g for 5 min. Cell viability was determined as described in Section 2A.5 and was always over 70%.

AM-enriched BAL cells were prepared by adherance to plastic tissue culture flaks (Nunc) according to the method of Koretzky et al. (1982). BAL cells were cultured in warm (37°C) RPMI-Mac medium (RPMI-1640, 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone), at a concentration of $1x10^6$ cells/ml, at 37°C in an atmosphere of 5% CO_2:95% air in tissue culture flasks kept in an horizontal position, the cap loosely fitting. The adherent cell population was harvested by removing the non-adherent cells, washing the adherent cells with sPBS and recovering them with sterile phospate buffered saline (sPBS) containing 0.02% ethylenediaminetetraacetic acid (EDTA) and incubating at 37°C for 15 min. Cells were removed from the flask by vigorous pipetting. Cell yield and viability was determined as described in Section 2A.5. Harvested cells were washed twice with sPBS before extracting the RNA. Alternatively, total RNA was isolated directly from the adherent cells as described in section 2B1.1.

2A.2 Afferent Lymph Cells

Afferent lymph cells were obtained by the chronic cannulation of the prefermoral psuedo-afferent lymph ducts (Hall, 1966; Hopkins et al., 1985). Briefly, the prefermoral lymph node was surgically removed. Within six to eight weeks the afferent lymphatics reanastomose with the single efferent duct which remained intact at removal of the lymph node. All surgery was performed by Professor J. Hopkins, (Edinburgh University). Afferent lymph was collected into sterile siliconised plastic bottles containing 400 U of heparin, 1000 U penicillin and 1 mg streptomycin, and, where indicated, actinomycin D (Sigma Aldrich) to a final concentration of 1 μg/ml. Collections were performed at 24 hour intervals to record lymph volume and cell counts. To obtain cell populations as near to physiologically normal, animals were allowed at least seven days postoperative recovery and lymph assessed to be absent of erythrocytes and inflammatory cells (polymorphonulear cells [PMN]) using flow cytometry (Section 2B.8).

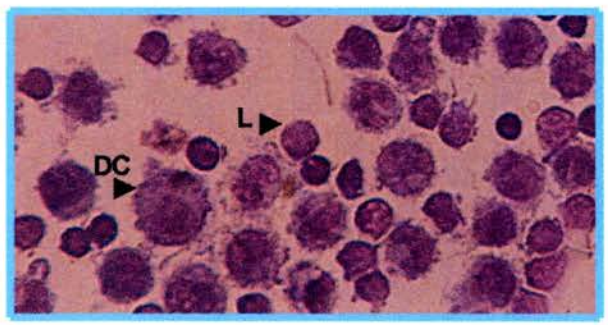
2A.3 Enrichment of Afferent DC and AM.

Afferent lymph and BAL were enriched for DCs and AM, respectively using centrifugation through a stepped sucrose gradient (Optiprep™ [Axis-shield]). During experiments evaluating cytokine expression, the buffers were supplemented with 1 μg/ml actinomycin D (Sigma Aldrich). Cells were washed in separation buffer (HBS, 0.5% bovine serum albumin [BSA], 1 mM EDTA), harvested and re-suspended in the same medium. Cell suspensions did not exceed 2 $x10^7$ cells/ml, volumes of 2.5 mls or 10 mls were transferred to 50 ml tubes (Nunc). Optiprep was added to the cells to a final concentration of 29% v/v, and mixed by swirling. These were overlaid with 4 ml or 7.5 ml 25% v/v Optiprep prepared in separation buffer respectively, followed by 10 ml or 20 ml 20% v/v Optiprep respectively, then 1ml HBS. The gradient solutions were centrifuged at 600 x g for 25 min at room temperature (rt), no brake was applied on deceleration. DCs and AM were collected from the interphase of 20% Optiprep and HBS, and washed in twice in sHBSS. Proportions of DCs or AM were consistently greater than 65% or 80% respectively as determined by cell morphology. Morphological analyses are shown in Figure 2.1.

2A.4 Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were utilised for titrating monoclonal antibodies (mAb) used in flow cytometry experiments (2B.8.5). Blood was collected from the jugular vein into 50 ml tubes (Nunc) containing 5 U/ml heparin and diluted by addition of an equal volume of sPBS. The diluted blood was layered onto an equal volume of rt Lymphoprep (Axis-shield) and centrifuged at 870 x g for 20 min at rt, with no break applied on deceleration. PBMCs were collected from the Lymphoprep:PBS interphase and washed twice in sPBS before cell surface immuno-labelling for flow cytometric analysis.

a)



b)

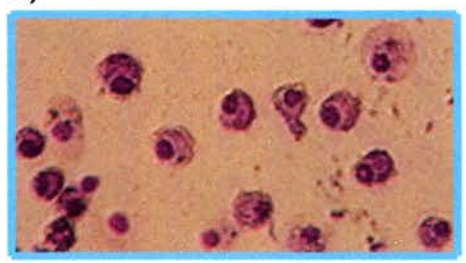


Figure 2.1 Morphological analysis of DC and AM -enriched cell preparations.

Dendritic cells (DCs) or alveolar macrophages (AM) were enriched from freshly isolated cell preparations as described in Section 2A.3. Cells were prepared on glass slides using a cytospin technique and morphology visualised using basic nuclear / cytoplamic staining (Section 2A.6).

a) Afferent lymph cells were enriched for DC. Cell types are indicated: DC: Dendritic cells, L: Lymphocytes. DC possessed an invaginated plasma membrane, with a large round nucleus and a high nuclear:cytoplasmic ratio. In comparison, lymphoyctes (L) were smaller, spherical, dense cells. Using these criteria DC comprised >65% of the cells. Original magnification x 1,250.

b) Bronchoalveolar cells enriched for alveolar macrophages. Alveolar macrophages AM are indicated with an arrow. AM had a highly vacuolated appearance with a large eccentric nucleus that was slightly indented. AM comprised >80% of the cells. Original magnification x 625.

2A.5 Cell Viability and Counting

Cell viability was determined using trypan blue exclusion. Cells were suspended in 0.1% trypan blue (Sigma Aldrich), and counted in a haemocytometer. The number of unstained viable cells was counted per 1 mm square. The cell count represented the number of cells x 10^4 / ml of cell suspension.

2A.6 Cytospin Preparation and Staining

Morphological analyses of fractionated or un-fractionated cells were carried out by preparing cytospins of single cell suspensions. 50 µl of cell suspension ($1x10^6$ cells/ml) was spun onto pre-chilled silane-prep™ microscope slides (Sigma Aldrich) at 300 rpm for 3 min using a Cytospin-2 (Shandon). Cytospins were fixed in ice cold acetone for 5 min and stained by covering in Gurr® Giemsa's stain solution (improved R66, Merck) for 15 min. Following staining, cytospins were washed, dried and covered with a cover-glass using clear nail varnish as a mounting medium.

2B Immunocytochemistry

2B.1 Monoclonal Antibodies

The mAb used in this thesis are listed in Appendix 3. This illustrates their isotype, specificity, source and working dilutions for immuno-labelling and flow cytometric analysis. The mAb were obtained from in house stocks (John Hopkins, Edinburgh University), clones purchased from the European Collection of Cell Cultures (IL-A24), or kind gifts of Dr. Jan Naessens, (International Livestock Institute; Nairobi) (3.29B1, ILA-156, ILA-159, ILA-190). mAb specificity was confirmed by analysing reactivity with afferent lymph cells (ALC) or PBMC using flow cytometry.

2B.2 Production of Saturated Culture Supernatants

In some instances B hybridoma monoclonal cell lines were grown in culture and the culture supernatant rich in monoclonal antibodies harvested. The hybridoma cells were stored under liquid nitrogen in 1 ml aliquots and were recovered by

thawing in a water bath at 37°C for 2 min. Cell suspensions were gradually added to 20 mls warm (37°C) culture medium (RPMI-1640, 20% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate). Cells were collected by centrifugation at 400 x g for 5 min, re-suspended in 5 mls culture medium and maintained at 37°C in an atmosphere of 5% CO_2:95% air, culture flask caps loosely fitting. Hybidoma cells grew as non-adherent aggregates and were passaged when required. Once the cells were established in culture, FCS content in the culture medium was reduced by half with each passage and replaced with Ultradoma PF serum free medium (BioWhittaker). The saturated culture supernatant was harvested from passage 5 onwards after removing the cell debris at 1500 x g for 15 min, and stored at –20°C in the presence of 0.01% sodium azide.

2B.3 Purification of Ascitic Fluid and Saturated Supernatant

Immunoglobulin (Ig) from ascitic fluid or hybridoma supernatant was purified by ammonium sulphate precipitation according to the method of Hardy (1986). Briefly, Ig was precipitated with ammonium sulphate (pH 6 – 7) at 45% saturation (volume / volume [v/v]) for 30 min at rt. The preparations were centrifuged at 10 000 x g for 20 mins. Solidified precipitate was washed twice with saturated ammonium sulphate and re-dissolved in PBS. For dialysis, cellulose tubing (Fisher) was cut into strips and boiled in 0.1 mM EDTA for 5 min, washed in distilled H_2O and boiled for a further 5 mins in 0.1 mM EDTA. The strips were washed again and stored in distilled H_2O at 4°C until required. Dissolved Ig was extensively dialysed into PBS for 12 hours at 4°C. The protein concentration was determined by spectrophotometry OD_{280}, with an absorbance reading being equivalent to 0.715 mg/ml protein. mAb were stored at –20°C in the presence of 0.01% sodium azide.

2B.4 Biotinylation of Monoclonal Antibodies

Ig solutions were adjusted to 1 mg/ml by dilution or concentration using a protein G-capture column (HiTrap™ Protein G Sepharose™ HP, Amerhsam PB), following the manufacturers instructions. Ig's were exchanged into 0.1 M $NaHCO_3$ (pH 8.3) using a Sephadex G 25 (PD-10) column (Amersham PB) to ensure no sodium azide remained in the solution, as this would inhibit down-stream biotin

conjugation. Biotinylation was then carried out by incubating the Ig (at 1 mg/ml) for 5 hours with biotin (biotin-amido caproate N-hydroxysuccinimide ester) in dimethylsulphoxide (DMSO) (2.5 mg/ml) at a biotin to protein ratio of 75 μg : 1 mg. Biotinylated Ig was then extensively dialysed against PBS, 0.01% w/v sodium azide, ph 7.2 for 12 hours at 4°C before a final 3 hour dialysis against PBS-azide containing 20% v/v glycerol. Biotinylated mAbs (in 20% glycerol) were stored at –80°C.

2B.5 Polyacrylamide Gel Electrophoresis (PAGE)

When new mAb preparations were used, Ig content was analysed by fractionating the proteins by polyacrylamide gel electrophoresis (PAGE) and visualised by staining or western blotting. Proteins were fractionated by discontinuous PAGE through Tris-glycine buffer containing SDS (255 mM glycine, 0.1% SDS, 50 mM Tris HCl). Gels comprised of a stacking gel and a resolving gel. The resolving gel consisted of 12% w/v acrylamide, 375 mM Tris pH 8.8, 0.1% w/v SDS, 0.5% w/v ammonium persulphate (APS) and 0.5% TEMED. The stacking gel consisted of 3% w/v acrylamide, 125 mM Tris pH 6.8, 0.1% w/v SDS, 0.5% w/v APS, 0.1% w/v TEMED. Samples were boiled for three min at 100°C in sodium dodecyl sulphate (SDS) sample buffer (Appendix 1) before electrophoresis through vertical slab gels using Bio-Rad Mini Protean II gel equipment. Gels were run at 140 Volts for 1.5 hours or until the dye front reached the bottom of the gel. Bio-Rad low molecular weight markers (range 111 – 20.5 kd) were run on each gel.

2B.6 Staining of SDS-PAGE Gels

Gels were stained in 0.25% Coomassie brilliant blue-R in 50% v/v methanol, 10% v/v acetic acid for 30 mins. Gels were then destained in several changes of 20% v/v methanol, 10% v/v acetic acid and dried onto wet 3 mm filter for 2 hours at 80°C on a vacuum drier (Biorad Labs. gel drier).

2B.7 Western Blot

Western blot was used for the visualisation of fractionated Ig that was obscured by serum proteins of approximately 60 kDa, and could not be visualised by Coommassie staining. After electrophoretic separation on SDS-PAGE, proteins were

transferred onto nitrocellulose membrane (Hybond-C, 0.45 μm, Amersham PB PB) at 0.8 mA per cm^2 for 45 min in buffer containing 48 mM Tris base, 39 mM glycine, 0.037% w/v SDS and 20% v/v methanol. The nitrocellulose membrane was washed several times with H_2O followed by PBS, then blocked with a solution of PBS, 5% w/v marvel non-fat dried milk powder, 0.2% v/v tween20® and 0.02% w/v sodium azide. Blocked membranes were washed several times with PBS and incubated for 1-4 hrs with primary antibody then washed several times in PBS. Membranes were then incubated with second antibody or substrate for 1 hour, washed several times in H_2O and air-dried.

2B.8 Flow Cytometry

For phenotypic analysis, cell preparations were washed twice in FACS buffer (PBS, 2% w/v BSA, 0.1% w/v sodium azide, pH 7.2, filter sterilised) and cell concentration adjusted to 10^6/ml for immuno-labelling of cell surface antigens.

2B.8.1 Single Immuno-labelling

To block non-specific binding, cells were incubated with 1% normal lamb serum (NLS) for 10 mins on ice before being washed twice with FACS buffer. Because afferent DC were the target cell population in these experiments, 10 X the number of ALC than ALC-enriched DC were labelled (DC comprise 1-5% ALC). $5x10^5$ ALC or PBMC or $5x10^4$ DC were incubated for 30 mins on ice with 50 μl mAb diluted appropriately in FACS buffer (Appendix 3). Unbound antibody was removed by washing twice with 1 ml FACS buffer. The cells were then incubated with 50 μl florescein isothiocyanate (FITC) -conjugated anti-mouse Ig (Sigma Aldrich) in the dark for 30 mins on ice. Cells were washed three times in FACS buffer, and re-suspended in FACS buffer or fixed with 2% w/v paraformaldehyde and stored at 4°C overnight before analysis. The control samples were treated with 1:500 normal mouse serum (NMS) (Scottish blood transfusion service) instead of primary antibody.

2B.8.2 Double Immuno-labelling

For the staining of two surface molecules using mAbs from different species, cells were incubated with NLS and dispensed as above and incubated simultaneously with 25 µl of each mAb (one of which was biotinylated). They were incubated and washed twice in FACS buffer as above. The biotinylated primary mAb was rat Ig (SW73.2, Appendix 3), and was detected with streptavidin-phycoerithrin (SA-PE) (Sigma Aldrich). Other primary antibodies were detected with a species-specific FITC-conjugated second antibody (goat anti-mouse IgG Fab specific) (Sigma Aldrich). Cells were incubated with 25 µl each of SA-PE and Ig-FITC in the dark for 30 mins on ice. Cells were washed and prepared for flow cytometric analysis as above. Negative controls were stained with 1:500 NMS and 1:100 biotinylated normal rat serum (NRSbio) or biotinylated (NMSbio) as first antibodies depending on the species (mouse or rat) in which the primary mAb were raised.

For double staining using antibodies raised in the same species, cells were stained separately with each antibody; first with the mAb to be labelled with FITC. After incubation, cells were washed twice and then incubated with FITC-antiglobulin conjugate as above and washed three times. Cells were then stained with the second biotinylated antibody prior to labelling with SA-PE as above.

2B.8.3 Flow Cytometer Settings

The immunofluorescence studies described were carried out using the FACScan, or FACScaliber cytometers (Becton Dickinson). Different cytometers were used during the project depending on the availability of machines. Data was analysed using the WinMDI software. In each case data was derived by analysing 10 000 cells (or events) per sample. Duplicate or triplicate samples were used in each experiment. After staining, the fluorescence measurements were made on homogenous populations of cells by setting gates on cell size and complexity dot plots (Figure 2.2) that were distinguished using appropriate linear photo multiplier tube (PMT) voltage amplifications for forward (FSC) and side (SSC) scatter parameters excluding unwanted cells. These FSC and SSC parameters define cell

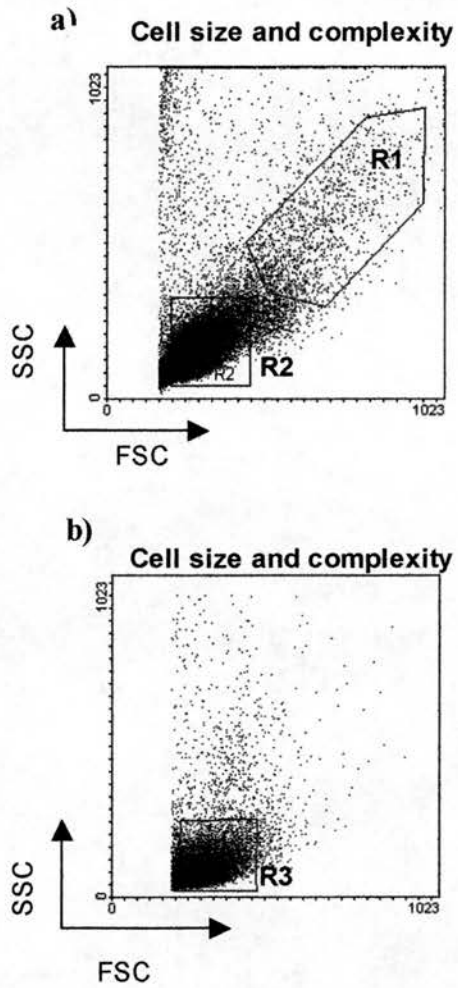


Figure 2.2 Gates used in flow cytometric analysis. Freshly isolated afferent lymph cells or peripheral blood mononuclear cells were prepared for flow cytometric analysis as described in Chapter 2. Cell populations were defined by light scatter properties.

a) Afferent lymph cell populations. Dendritic cells were defined by high forward scatter (FSC) and high side scatter (SSC) (R1) and lymphocytes with low to medium FSC and low SSC (R2).

b) Peripheral blood mononuclear cells (R3)

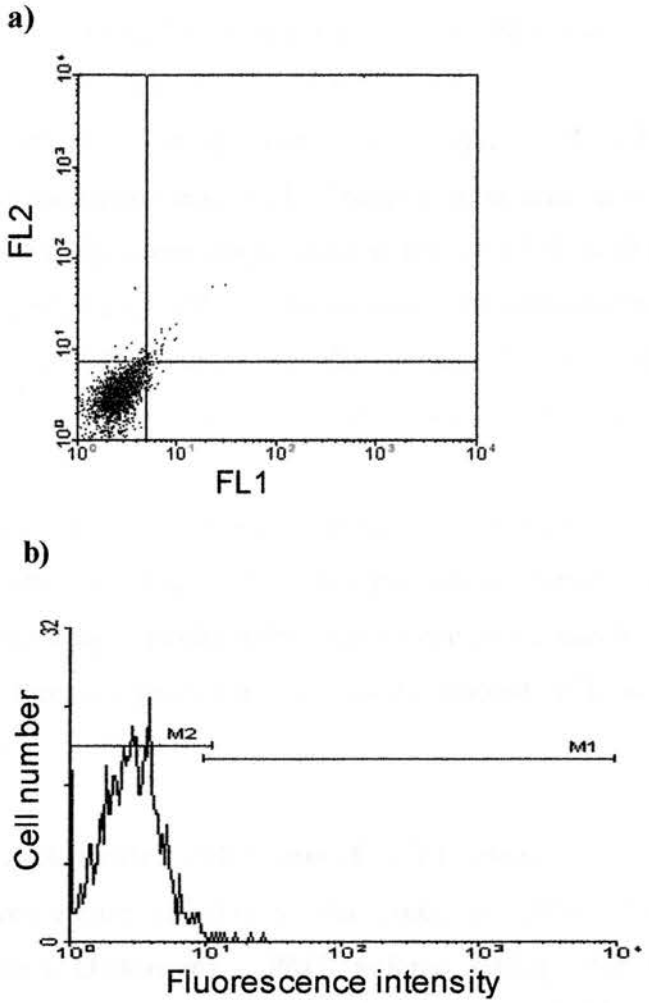
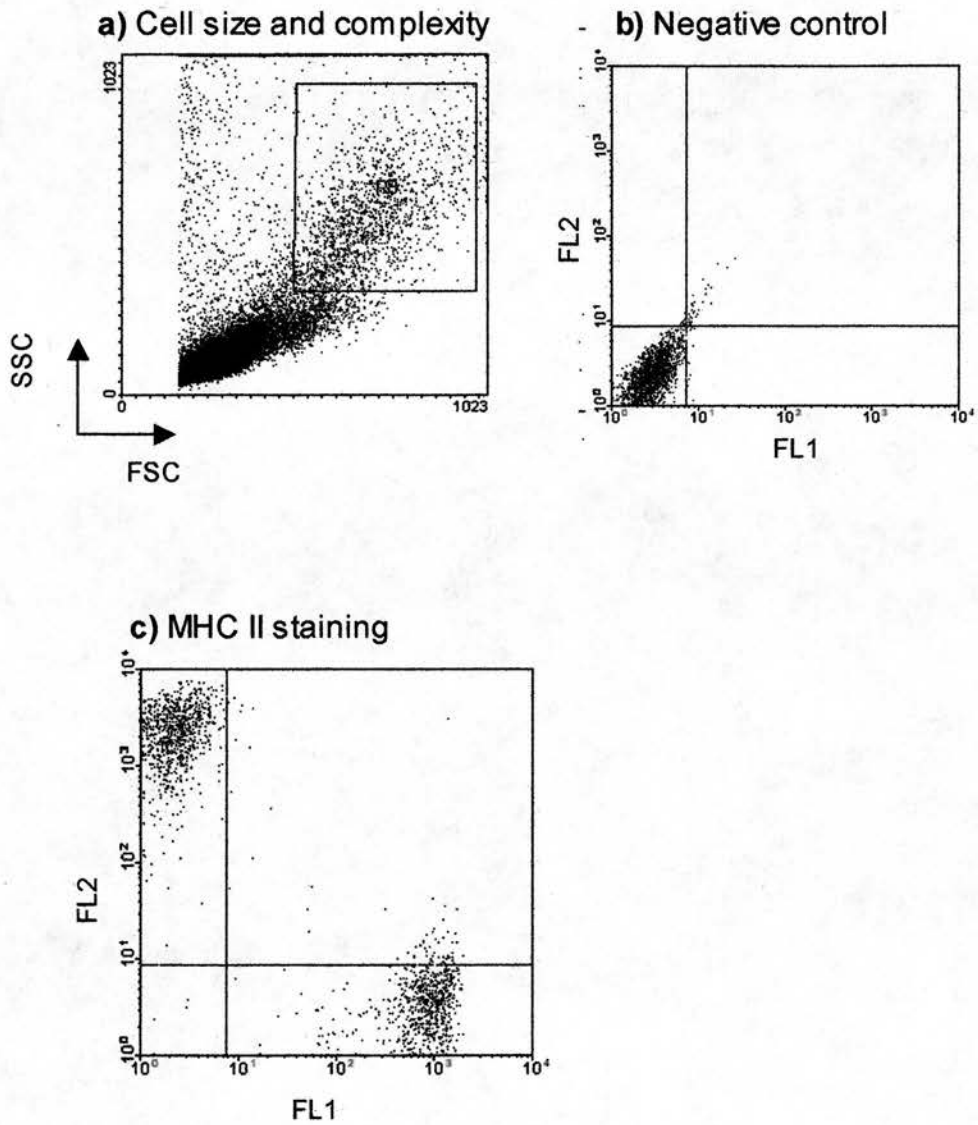


Figure 2.3 Cell phenotyping negative controls. Cells were stained with 1:500 NMS or 1:100 NMSbio and secondary conjugates to determine the background fluorescence for FL1 and FL2 parameters, respectively.

a) Negative control cells. The quadrants were adjusted so 99% of the cells were in the lower left quadrant.

b) Marker (M1) was set to determine the percentage positive cells in relation to 1% of negative control cells.


a) Cell size and complexity
SSC
FSC
1023
0
1023
b) Negative control
FL2
FL1
c) MHC II staining
FL2
FL1

2B.8.5 Titration of Monoclonal Antibodies

The mAbs, conjugates and secondary reagents used in the flow cytometry experiments were titrated to establish optimal working conditions. Sheep ALC and DC were stained with various dilutions of mAb preparations. A range of primary mAb incubation times was tested (20 mins – 45 mins), maximal staining was evident after 30 min incubation on ice (not shown). Purified antibodies and fluorochromes (SA-PE and Ig-FITC) were tested at a range of concentrations, live cell populations were gated and fluorescence analysed. The optimal working dilution of each of the secondary reagents chosen was which gave the strongest fluorescence intensity and clear distinction between the positive and negative populations within the normal range. The optimal working dilution of the primary antibody giving strongest specific fluorescence and lowest background staining of the negative control cells was chosen. An example is shown in Figure 2.5.

2B.9 Negative Selection of DC: Magnetic Activated Cell Sorter (MACS).

Afferent lymph lymphocyte depletion protocols involved mAb staining and separation using a magnetic activated cell sorter (MACS) (Miltenyl Biotec). DC enriched afferent lymph cells (Section 2A.3) were washed twice in buffer 1 (PBS, 1% w/v BSA, 0.05% w/v sodium azide, 5μM EDTA) and $5x10^5$ cells incubated with 50 μl of biotin-conjugated mAb at optimal staining concentration for 30 min at 4°C. Un-bound antibody was removed by washing the cells twice in buffer 1, the cells were then labelled with 10 μl strepatividin-coated microbeads (Miltenyl Biotec) in 100 μl with buffer 1 for 20 min at 4°C. Cells were washed as above and re-suspended in 500 μl buffer 2 (PBS, 0.5% w/v BSA, 2mM EDTA) before magnetic selection using an equilibrated vario MACS column (Miltenyl Biotec) fixed in a magnetic stand. The column was washed with 2 mls buffer 2 and the elute collected as the negative cell fraction. After removing the column from the magnet, the positive cell fraction was eluted with buffer 2.

The cell fractions were analysed by flow cytometry. The un-selected (negative) cell fraction should be absent of cells expressing markers used for magnetic separation, ie. lymphocyte-specific markers. Cells from the negative fraction were stained with

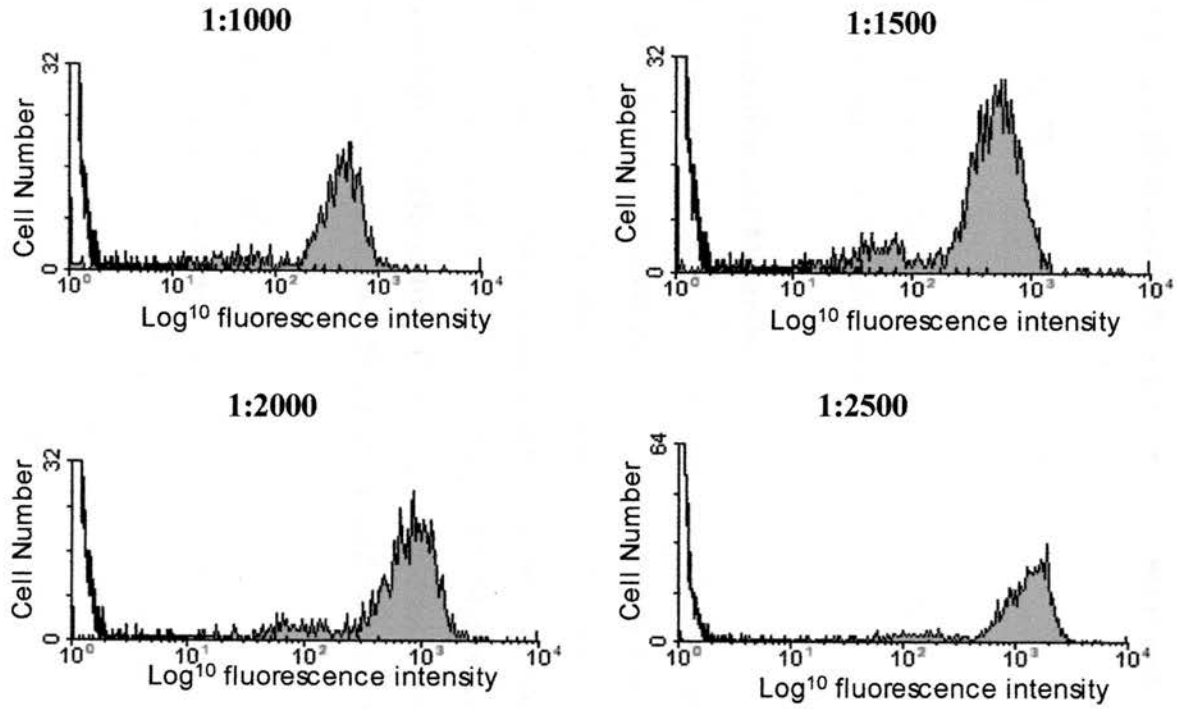


Figure 2.5 Titration of mAb SW73.2:bio The optimal concentration for each mAb was determined for analysis of DC (Chapter 5). An example is shown. $5x10^5$ afferent lymph cells were stained with 50 µl SW73.2 biotin conjugate at various concentrations and S:PE (1:50). mAb concentrations are indicated with each profile. Dendritic cells were gated on high forward and side scatter, and florescence profiles analysed.
Saturation of mAb binding was determined by the histogram profile showing normal distribution; here at a concentration of 1:1500, which was used in subsequent experiments.

the mAb used in MACS selection as described in Section 2B.8.2. The positive cell fraction should only contain cell expressing the markers used for selection, and as such be 100% positive. Biotin-conjugated mAb were used for the primary antibodies and were labelled with biotin-coated beads. This labelling does not engage all free biotin-streptavidin binding sites, so bound primary mAb conjugates can be directly labelled with SA-PE and analysed by flow cytometry. Cells from the positive fraction were incubated with SA-PE (Sigma Aldrich) at 4°C for 30 mins, and washed twice. Cells were analysed on a FACSscan (Becton Dickinson).

2C Molecular Biology

All DNA sequencing was carried out by Mr. I. Bennet (University of Edinburgh) on a LI-COR DNA sequencer (model 4000L)

2C.1 RNA Extraction and Methods

Precautions were taken to preserve the integrity of RNA transcripts throughout the following procedures. Particular emphasis was placed on avoiding contamination with RNases. Commercially available RNase free solutions and sterile nuclease-free plastic wear were used. Glass wear was treated with RNase ZAP (Sigma Aldrich), and rinsed with nuclease-free H_2O (Sigma Aldrich). Gloves were changed frequently. Nuclease-free H_2O (Sigma Aldrich) was used to prepare solutions. Solutions were stored at –20°C and retained on ice throughout use.

2C.1.1 Isolation of Total RNA

Total RNA was isolated using one of two methods:

For less than $5x10^6$ cells, SV Total RNA Isolation System (Promega) was used following the manufacturers instructions.

For more than $5x10^6$ cells RNAzol B (Biogenesis) was used to extract RNA. Adherent cells in tissue culture flasks were washed twice with PBS then lysed directly by addition of RNAzol B to the flask. Non-adherent cells were washed twice, the medium removed then re-suspended in RNAzol B at 1 ml per $5x10^7$ cells. Following lysis, 0.1v of chloroform was added, samples vigorously shaken and

stored on ice for 5 min. Samples were centrifuged at 12000 x g for 15 min, the upper aqueous phase transferred to a fresh tube and an equal volume of ice-cold isopropanol added. Precipitation was carried out for 30 min on ice, or overnight at 4°C. The samples were then centrifuged at 12000 x g for 15 min. RNA pellets were washed with 75% ethanol, air dried then re-suspended in nuclease-free H_2O. To ensure complete removal of cellular DNA, samples were treated with DNase I. RNA (1 - 10 μg) was incubated with 10 U of RQ1 RNase-free DNase (Promega) at 37°C for 30 min. The sample was then extracted with phenol/chloroform/isoamyl alcohol (pH 4.7, 25:24:1) (Sigma Aldrich), followed by chloroform/isoamyl alcohol (24:1, Sigma Aldrich), and precipitated with 0.5v of 7.5M NH_4 acetate at –70°C for 30 min. Following centrifugation at high speed for 5 min, the RNA pellet was washed and re-suspended as previously mentioned. If not for immediate use, RNA samples were stored in nuclease-free H_2O at –70°C.

2C.1.2 RNA Analysis

The concentration of RNA samples was determined by spectrophotometry with an OD_{260} of 1.0 being equivalent to 40 μg/ml RNA. Absorbance readings at A_{280} were also measured to check sample purity; pure RNA preparations have a A_{260}/A_{280} ratio of >1.9.

Gel electrophoresis was also used to demonstrate the intact nature of the RNA. 28S and 18S bands were required to indicate that the RNA had not undergone degradation. Approximately 2 μg of RNA was denatured by heating to 80°C for 3 min then chilled on ice for 3 min, and then run on a 1% agarose gel (Section 2C.3.4), 1 μg of the EcoR I/Hind III digested lambda DNA markers was run on each gel. Gels were visualised under ultra violet (U.V.) light.

Where indicated, RNA was analysed using the RNA 6000 Nano assay system (Agilent Technologies) to demonstrate the intact nature of the RNA samples. Samples containing 5 – 500 ng RNA were analysed according to the manufacturers instructions.

2C.2 cDNA Synthesis

Unless otherwise indicated, cDNA synthesis was carried out using Superscript II RNase H⁻ Reverse Transcriptase (RT) (Invitrogen LT) according to the manufacturers instructions. Oligo $(dT)_{15}$ primer (Promega) were used to initiate the cDNA synthesis from total RNA as follows; 1 μg of total RNA plus 500 ng oligo-dT was made up to 12 μl with H_2O, heated to 70°C for 10 min then chilled on ice. The following constituents were then added; 4 μl of 5 X transcription buffer (final conc. 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 20 mM dithiothreitol and 100 μM of each dNTP. The mixture was incubated at 42° for 2 min prior to addition of 200 U RT and incubation at 42°C for 50 min. The reaction was then inactivated by heating at 70°C for 15 min. Negative controls were included for genomic DNA contamination; cDNA synthesis was carried out omitting reverse transcriptase enzyme. Theses samples were then analysed by PCR (Section 2C.4).

2C.3 Rapid Amplification of cDNA Ends (RACE)

cDNA synthesis was also carried out to allow the 'rapid amplification of cDNA ends' (RACE) using the 5'/3' RACE system (Roche). This technique utilised the 3' polyA-tail of eurkaryotic mRNA for cDNA syntheis directed by an oligo (dT)-anchor primer. The 5' region was obtained by synthesising cDNA with a specific anti-sense primer. After purification, the cDNA was polyA-tailed, and amplified by PCR using the oligo (dT)-anchor primer. Anchor primers were used in subsequent PCR reactions. The protocol is as follows: for the synthesis of cDNA, final reaction volumes of 20 μl consisted of 1 μg total RNA, 250 pM specific primer or 1.2 μM oligo (dT)-anchor primer, 100 μM of each dNTP, 1 X reaction buffer (50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol), and 20 units AMV RT. The reaction was incubated at 55°C for 1 hour, and heat inactivated at 65°C for 10 min. For amplification of the 5' region, cDNA synthesised in the presence of the specific primer was purified using the High Pure PCR product purification system (Roche), following the manufacturers instructions. The purified cDNA was then subject to a polyA-tailing reaction that consisted of 19 μl cDNA, 2.5 μl 2 mM dATP, and 2.5 μl 10 X buffer (final concentration 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM

KCl, pH 8.3). The reaction mixture was incubated at 94°C for 3 min, and briefly chilled on ice before the addition of 10 U terminal transferase and incubation at 37°C for 30 min. The reaction was heat inactivated at 70°C for 10 min.

2C.4 Polymerase Chain Reaction (PCR)

Care was taken throughout the preparation of PCR reactions to avoid contamination of samples with amplified products, or unwanted DNA. Gloves were worn and changed regularly, and sterile tubes and aerosol resistant tips were used throughout the procedure. All PCR reagents were aliquotted and stored in smaller volumes.

PCR reactions were carried out in sterile 0.2 ml thin walled tubes with final reaction volume of 50 µl. The basic components of the PCR reaction were as follows. 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl pH8.3, 1.5 mM $MgCl_2$), 200 µM of each dNTP, DNA template (2 µl of a cDNA synthesis reaction or 10 ng pDNA), 10 – 50 pmoles of each primer. These constituents were mixed then collected by pulse high speed centrifugation. To avoid ambiguous primer binding, 'hot start' PCR was used; reaction tubes were heated to 94°C for 3 min prior to the addition of 1 unit of Taq (*Thermus aquaticus* DNA) polymerase enzyme. Different types of commercially available Taq were used in different experiments, manufacturers are listed in the relevant sections. Cycling was carried out in a Hybaid Sprint Thermocycler. Oligonucleotide primer sequences are listed in Appendix 4. Each primer pair was optimised for [$MgCl_2$], an example is shown in Figure 2.6. Cycling parameter varied according to the primers and as such are described in the relevant sections.

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2C.5 Agarose Gel Electrophoresis

Following PCR, 20% of the reaction was analysed on a 1 – 1.5% w/v agarose gel (Type I: low EEO, Biogene). Agarose was melted in TAE buffer (Appendix 1) containing 1 µg/ml ethidium bromide. Samples were mixed with 1/5 volume of 5 X DNA sample buffer, loaded and the gel run at 70-80V for 60-90 min. For DNA fragments 100 b.p. – 1 k.b., 100bp. DNA ladders (MWG Biotech) were used; DNA fragments > 1 k.b. EcoR I/Hind III digested lambda DNA markers were used, 1µg of

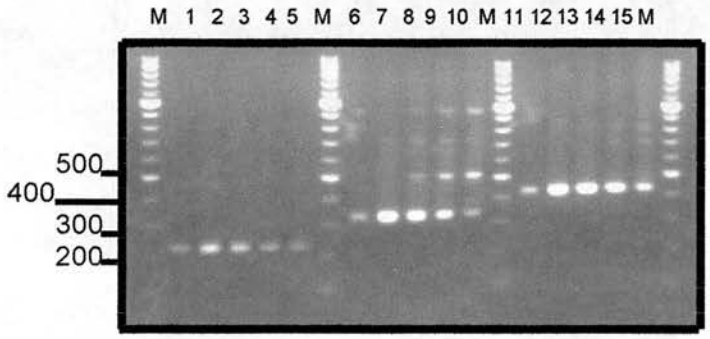


Figure 2.6 $MgCl_2$ optimisation for PCR primers. $MgCl_2$ concentration was optimised for each primer pair used in this project, an example is shown;

Lanes 1-5 IL-6 (Primers X68723F & X68723R; Appendix 4)

Lanes 6-10 IL-12 p40 (Primers AF004024F2 & AF004024R; Appendix 4)

Lanes 11-15 IL-10 (Primers Z29362F & Z29362R; Appendix 4)

PCR reaction [$MgCl_2$] was 0.5 mM lanes 1, 6, 11;

1.5 mM lanes 2, 7, 12

2.5 mM lanes 3, 8, 13

3.5 mM lanes 4, 9, 14

4.5 mM lanes 5, 10, 15.

Templates were 10 ng plasmid DNA containing the appropriate cytokine cDNA sequence. Reactions were incubated for 30 cycles of amplification (94 1'; 55 1'; 72 1') followed by 1 extension cycle (72 7'). Optimal $MgCl_2$ concentration for each primer set was 1.5 mM.

the marker was run on each gel. DNA concentration was determined using spectrophotometry, with an OD_{260} of 1.0 being equivalent to 50 μg/ml DNA. Absorbance readings at A_{280} were also measured to check sample purity; pure DNA preparations have a A_{260}/A_{280} ratio of over 1.7.

2C.6 Purification of DNA

Vector DNA or DNA amplified by PCR which were to be subsequently used for cloning were subject to one of the two purification methods:

DNA fragments excised from agarose gels were purified using a DNA:agarose-gel extraction kit following the manufacturers instructions. Following purification the concentration of the DNA sample was determined using spectrometry.

Single PCR products, or linearised vector DNA were purified using spin-bind column (QiAquick®, Qiagen) following the manufacturers instructions. To check the procedure 10 μl of the sample was run on an agarose gel, and the concentration of the DNA sample determined using spectrometry.

2C.7 Cloning of PCR Products

PCR products which had been purified as described above were cloned using the pGEM®-T Easy vector system (Promega) that utilises TA cloning. Briefly, ligations were set up as 3:1 to 1:3 molar ratio of vector : PCR insert. Competent JM109 cells (Promega) were then transformed with 2 μl of the ligation reaction and the transformed cells were plated onto LB/ampicillin/X-gal plates (Section 2C.9) and incubated overnight at 37°C. Transformed colonies were identified by blue/white selection and positive clones were picked and grown overnight in 10 ml LB containing 50 μg/ml ampicillin.

2C.8 Competent Cells

The *E. coli* strains used in these studies were JM109 (TA cloning kit) or DH5α that were made competent according to the method of Inoue et al. (1990). Single colonies were picked into 10 ml SOB medium (Appendix 1) and grown for 5 hours then 2.5 ml of the culture diluted to 250 ml and grown to midlog OD_{600}=0.6.

Cultures were rapidly chilled, the cells collected at 2800 x g for 15 min then re-suspended in 80mls ice cold TB buffer (Appendix 1) and chilled for 10 min. Cells were centrifuged again at 15000 x g for 10 min then gently re-suspended in 5 mls TB, 0.3 ml DMSO (high grade, Sigma Aldrich) before being aliquoted into pre-chilled eppendorf tubes (Nunc) and snap frozen in liquid nitrogen. Competent cells were then stored at –80°C.

2C.9 Bacterial Cultures

E. coli bacterial strains were cultured in Luria Bertani (LB) broth (Appendix 1). The medium was supplemented with ampicillin (100 μg/ml) since the vectors used in this study all contained ampicillin resistance gene. LB / agar plates were made up with 1.5% bacteriological agar supplemented with 50 μg/ml ampicillin and were stored at 4°C. When plating JM109 cells with the vector pGEM-T-easy, each 10 cm plate was supplemented with 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) and 0.5 μM isopropyl-β-thiogalactopyranoside (IPTG) to allow blue-white selection.

2C.10 Preparation of Plasmid DNA

Plasmid DNA for sequencing was isolated, depending on bacterial culture volume, using QIAprep-spin columns (Qiagen) or the QIAfilter plasmid midi kit (Qiagen) following the manufacturers instructions.

Plasmid DNA that was analysed for insert size was isolated as follows; 1 ml of overnight culture was centrifuged at 400 x g for 10 min and re-suspended in 25 mM Tris pH 8, 10 mM EDTA pH 8, 50 mM glucose. Cells were then lysed in 0.2 mM NaOH, 1% SDS for 5 min at rt. Precipitation of bacterial DNA was carried out by addition of 5 M potassium acetate, 19% glacial acetic acid, incubation on ice for 5 min and high speed centrifugation for 3 min. The supernatant was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 100% ethanol, washed in 70% ethanol and re-suspended in 20 – 50 μl TE buffer (Appendix 1). Any bacterial RNA was degraded using 0.5 μg RNase A (Sigma Aldrich) by incubating at 37°C for 30 min, and then at 70°C for 5 min to inactivate the reaction.

2C.11 Restriction Endonuclease Digestion of DNA Samples

Restriction endonuclease digests of plasmid DNA to allow analysis of inserts was carried out as follows. The reaction was typically carried out in a 20 µl volume containing 10 µl plasmid DNA, 10 units of enzyme and the appropriate enzyme buffer (New England Biolabs). Digests were carried out at 37°C for 1-2 hours. Following digestion, inserts were analysed by running 10 µl of the reaction on an agarose gel (as in section 2C.3.4.).

2C.12 RNase Protection Assay (RPA)

An RPA was developed to analyse the expression of eleven ovine cytokines. Radiolabelled antisense RNA (riboprobes) were synthesised from a set of specific cDNA subclones, which were then hybridised with target RNA samples. Single stranded RNA species and excess probes are enzymatically digested and annealed species analysed by PAGE. A schematic illustrating the RPA methodology is shown in Figure 2.7. Inclusion of a probe for a housekeeping gene (GAPDH) enables normalisation between samples, and relative levels of hybridised RNA species can be calculated. As such, the RNase protections assay is a specific, highly sensitive, method for evaluating the levels of target mRNA. The development of the RPA is described in Chapter 5, the following sections describe the methodology of the RNase protection assay.

2C.12.1 Linearisation of Riboprobe Templates

Cytokine sub-clones were used as templates for riboprobe and positive control RNA synthesis, these are listed in Appendix 5. Plasmid DNA was linearised by restriction endonuclease digest, as described in Section 2C.8 and the samples then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) pH 7.5, followed by chloroform/isoamyl alcohol (24:1), washed in ethanol and precipitated with 3 M sodium acetate pH 5.2. Following high speed centrifugation for 15 min, DNA pellets were washed in 70% ethanol, air dried and re-suspended in 10 mM Tris pH 8.5.

2C.12.2 Preparation of Positive Control RNA

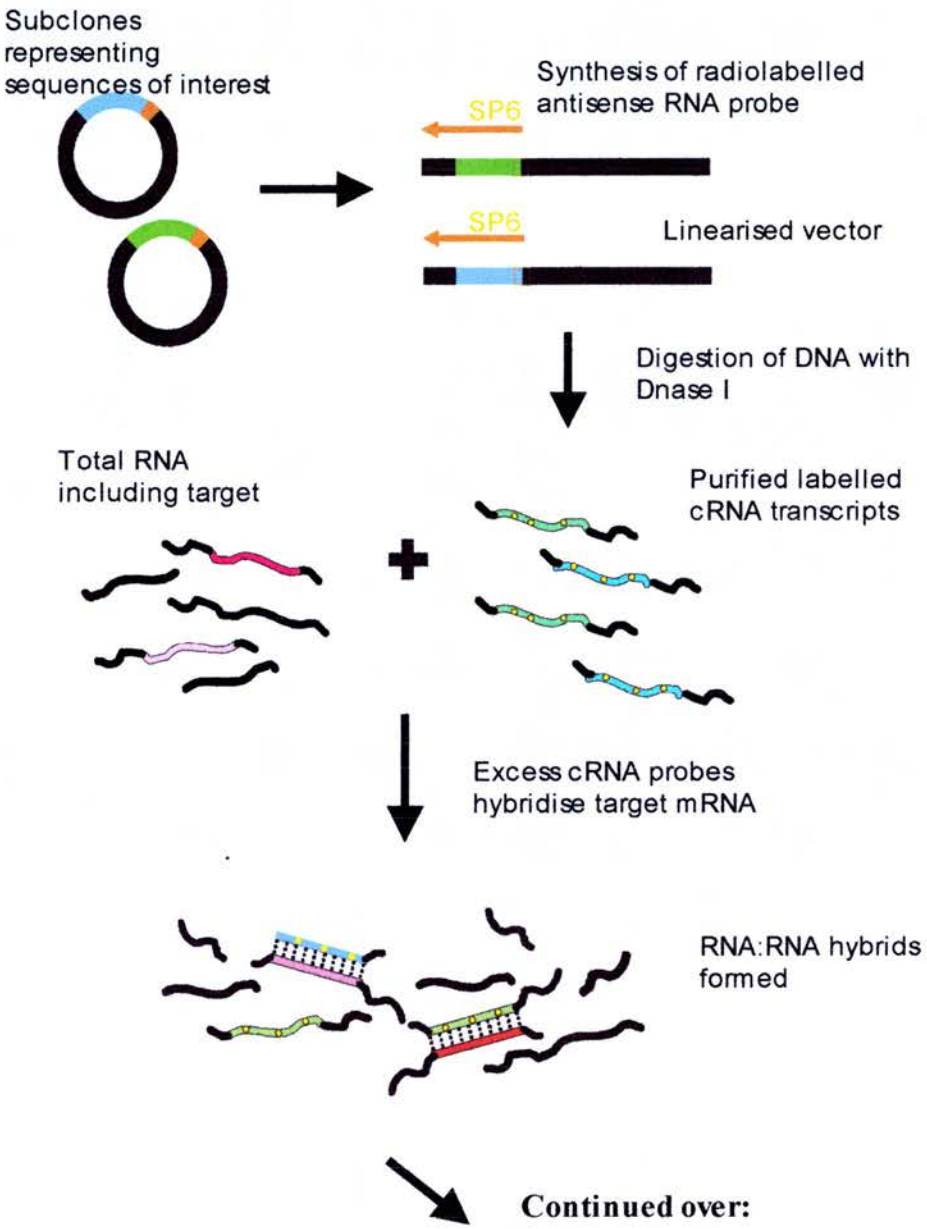
RNA was produced to use as controls for probe hybridisation. Transcription reactions were set up in 100 µl volumes containing 1-2 µg linearised template DNA, 100 mM DTT, 100 U RNasin (Promega), 2.5 mM each rATP, rGTP, rCTP, rUTP, 40 U T7 RNA polymerase and 1 X enzyme buffer, which were then incubated at 37°C for 2 hours. DNA template was removed by adding RQ1 RNase free DNase (1 U/µg DNA) (Promega), and incubating the sample at 37°C for 15 min. The sample was then extracted with TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1) pH 4.5 followed by chloroform /isoamyl alcohol (24:1), precipitated with 0.5 volume 7.5 M ammonium acetate in the presence of 2.5 volumes of 100% ethanol and incubated at –70°C for 30 min. After high-speed centrifugation for 20 min, the pellet was washed with 0.5 ml 70% ethanol, air dried and re-suspended in 100-200 µl H_2O.

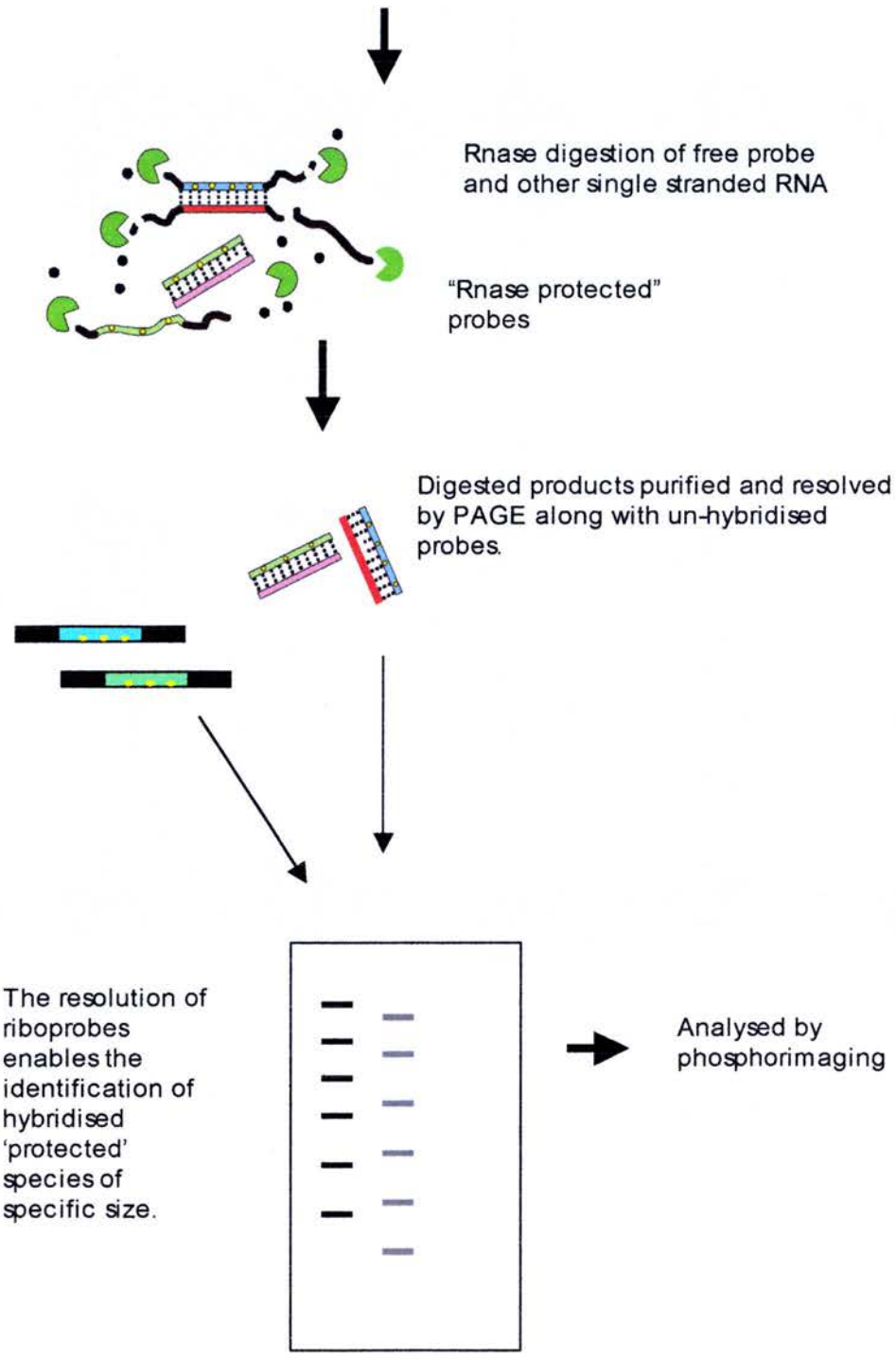
2C.12.3 Generation of Radiolabelled Probes

Antisense riboprobes were synthesised from a pool of cDNA templates (2C.12.1). Quantification of target mRNA is achieved when the riboprobes are at molar excess. So that the riboprobes would be present in the hybridisation mixtures at equivalent amounts, quantities were normalised to the GAPDH probe. The optimal quantity of GAPDH riboprobe to achieve riboprobe excess vs. cellular mRNA was 1.66 ng (Dr. A. Gossner; Edinburgh University). The quantity (ng) of template DNA required was determined by the formula; quantity of probe (ng) = quantity of vector (ng) x (insert size (kb.) / vector size (kb.)).

Unless otherwise stated, reagents used in probe synthesis were from Promega's Riboprobe Combination System SP6/T7. During synthesis, [^{32}P]UTP nucleotides were incorporated into the anti-sense RNA (riboprobe). The synthesis reaction was set up in a sterile tube containing lyophilised rUTP (10 µM), 2.5 mM each rGTP, rATP, rCTP and 120 µCi 5'-[α^{32}P] UTP (Amersham PB PB). The reaction was set up in a 10.5 µl volume containing an equimolar pool of linearised template DNA (Section 2C9.1), 10 mM DTT, 1.2 U RNasin, 20 U SP6 RNA polymerase and the enzyme buffer. Reactions were incubated at 37°C for 1 hour and terminated by adding 2 U RQ1-DNase and incubating at 37°C for 30 min.

Figure 2.7 The RNase protection assay. The RNase protection assay is a highly sensitive and specific method for the quantitation of mRNA species. The strategy for the development of a multiprobe RNase protection assay was to generate a series of ovine cytokine cDNA sub-clones. To generate probe templates the plasmids were linearised with an appropriate restriction endonuclease, purified, and combined in a set. The templates were used for RNA polymerase directed synthesis of ^{32}P-labelled anti-sense RNA (cRNA). The purified-labelled riboprobes were hybridised (at probe molar excess) with the target RNA. After hybridisation was completed, ribonucleases specific for single stranded RNA digested un-hybridised RNA and probe. The "RNase-protected" probes were resolved on a denaturing PAGE and were visualised by phosphorimaging. As the entire target mRNA was in solution and available for hybridisation the quantity of each mRNA species in the original RNA sample could be determined based on the intensity of the appropriately sized probe.


Subclones representing sequences of interest
Synthesis of radiolabelled antisense RNA probe
SP6
SP6
Linearised vector
Digestion of DNA with Dnase I
Total RNA including target
Purified labelled cRNA transcripts
Excess cRNA probes hybridise target mRNA
RNA:RNA hybrids formed
Continued over:


Rnase digestion of free probe and other single stranded RNA
"Rnase protected" probes
Digested products purified and resolved by PAGE along with un-hybridised probes.
The resolution of riboprobes enables the identification of hybridised 'protected' species of specific size.
Analysed by phosphorimaging

Radiolabelled riboprobes were purified after adding 4 μg yeast tRNA as carrier, using ProbeQuant G-560 spin columns (Amerhasm PB) following the manufacturers instructions. The quantity of incorporated probe was determined by adding 1 μl of the synthesis reaction to 1 ml HISAFE scintillation fluid (National Diagnostis), and using a β counter. Probes with an activity $>10^6$ cpm were used.

2C.12.4 Riboprobe:RNA Hybridisation

Total cellular RNA and positive control RNA were hybridised with specific riboprobes which bind by homologous interactions. Target RNA was lyophilised at rt for 1 hour in a DNA speed vacuum after incubation at –70°C for 15 min. After re-suspension in 8 μl hybridisation buffer (80% formamide, 1 mM EDTA, 400 mM NaCl, 40 mM PIPES, pH 6.7), 2 μl of the labelled probes were added to each RNA sample. Samples were vortexed and incubated at 90°C in a heated block, which was immediately turned down to 56°C overnight.

2C.12.5 Removal of Single Stranded RNA

Target mRNA species of interest were bound by complementary radiolabelled probes. These hybridised molecules are protected from enzymatic digestion using single-strand-specific RNase's which degrade 'un-protected' RNA. Following hybridisation, 100 μl RNase cocktail (10 mM Tris pH 7.5, 0.3 M NaCl, 5 μM EDTA, 33% v/v RNase cocktail™ (15 U RNase A, 606 U RNase T1) (Ambion) was added to the samples and incubated at 30°C for 45 min. The reaction was then inactivated by adding 18μl proteinase K (0.3% SDS, 600μg proteinase K) and 120 μg yeast tRNA (2 mg/ml) as a carrier, and incubating at 37°C for 15 min.

The samples were extracted with phenol: cholorform: isoamyl alcohol (25:24:1) pH 7.5, precipitated with 4 M ammonium acetate and 650 μ1 ethanol and centrifuged at high speed for 15 min. After the RNA pellets were washed in 100 μl 90% ethanol and air dried, each sample was re-suspended in 5μl loading buffer (80% formamide, 1 mM EDTA, 50 mM Tris-borate, pH 8.3, 0.05% w/v xylene cyanole and bromophenol blue), and stored on ice.

2C.12.6 Gel Resolution of Protected Probes

To quantify the abundance of hybridised radiolabelled probes, samples were resolved on denaturing acrylamide gels, and scanned with phosphorimaging. Each gel was prepared with 100 mls 5% acrylamide mix (National diagnostics) according to the manufacturers instructions. Immediately prior to casting, 300 µl 10% ammonium persulphate was added to polymerise the gel solution. Prior to assembly, sequencing gel plates were thoroughly cleaned, buffed with isopropanol, and one of the two siliconised. Plates were separated with 0.2 mm spacers, clamped and the gel poured at 10° angle. Once the well comb was inserted, the end of the gel was covered in clingfilm and cast overnight. Gels were run in TBE buffer and heated to 50°C before loading the samples. Prior to loading on a polyacrylamide gel, the samples were incubated at 90°C for 3 min then immediately chilled on ice for 3 min. Gels were run at 50°C at 80 to 85 Watts for 1.5 – 2 hours or until the leading dye front reached the bottom of the gel.

2C.12.7 Phosphorimaging

Gels were dried on 3MM filter paper on a vacuum drier, and exposed overnight against a phosphorimaging screen (Kodak). Screens were scanned on an imager (Molecular Dynamics).

2C.12.8 Densitometric Analysis

Data were collected after phosphorimaging scanning and pixel levels of radioactivity were analysed using Image Master 3D software (Pharmacia). The background levels in each lane were determined by the minimum level of density, and subtracted. This package allows the detection of bands of different intensities on the same gel image by adjusting the levels of contrast and intensity. The software automatically detected bands, and bands of interests were manually identified. Limits of band width were adjusted so that pixels of highest intensity were included; the same constraints were used for particular bands throughout the samples. Band volumes determined by the software package were presented as arbitrary values of pixel density. The values were exported into excel, and the ratio of intensity of each band (I_g) was calculated to the density level of the housekeeping gene GAPDH (I_{hg})

and represented as a 'normalised volume' according to the formula: $x = (I_g / I_{hg}) \times 100$. The normalised volumes were imported into GraphPad Prism version 3.00 for statistical analysis, and production of graphs. Individual data were used for analysis of the kinetics of cytokine gene expression. Mean values were determined by compiling the data obtained from separate donor animals that undergone similar experimental conditions.

2D Statistical Analysis

Data from individual experiments were pooled, and T tests (un-paired, two-tailed) and non-linear regression analyses were performed using the GraphPad Prism version 3.00 package. Results are expressed as mean ± standard error of the mean.

Chapter 3: Cloning and Sequencing of Sheep Interleukin-18

3.1 INTRODUCTION

Interleukin-18 (IL-18) was initially described as a factor produced in *Propionibacterium acnes* (*P. acnes*) primed, and LPS-challenged mice that induced high levels of IFNγ production above that of IL-12 alone, and was thus designated as IFNγ-inducing factor (IGIF) (Okamura et al., 1995a). IL-18 has subsequently been characterised in a variety of mammals and vertebrates. It is expressed by a wide variety of immune and non-immune cells including monocytes, macrophages and MoDCs (Stoll et al., 1998; Gardella et al., 1999). Studies using knock out mice demonstrated IL-18 as critical for the regulation of NK cellular and Th1-type immunity (Takeda et al., 1998), and augments CD3-stimulated T cell proliferation in a MLR (Yoshimoto et al., 1998). In addition, IL-18 enhances Fas ligand (FasL) (Okamura et al., 1995a) expression and cytotoxicity of natural killer cells (NK) and T lymphocytes (Hashimoto et al., 1999), which mediate IL-18 anti-tumour activity. IL-18 has also been shown to induce the production of tumour necrosis factor-α (TNFα) and IL-1β, the chemokines IL-8 and macrophage inflammatory protein-α (MIP-1α) (Puren, Fantuzzi and Dinarello, 1999; Fehniger et al., 1999; Netea et al., 2000), and enhance GM-CSF production (Micallef et al., 1996; Ushio et al., 1996).

Human IL-18 is a 18 kDa non-glycosylated protein and production of active IL-18 requires that its precursor (pro-IL-18) is processed by caspase 1 (Gu et al., 1997; Ghayur et al., 1997). The 5' region of the human IL-18 gene contains one LPS / phorobol 12-myristate 13-acetate (PMA) -regulated promoter and another constitutive promoter (Tone et al., 1997). The inducible promoter contains a number of transcription factor recognition sequences such as for activator protein-1 (AP-1) and Nuclear factor-kappa B (NFκB) (Tone et al., 1997).

3.1.2 Chapter Aims

At the outset of this project, sheep IL-18 cDNA had not been cloned. This cytokine plays an important role in regulating T cell responses, as well as participating in the early stages of cell-mediated immunity. It was therefore important to examine its expression by sheep DCs. This chapter describes the

cloning of ovine IL-18 by rtPCR and the rapid amplification of cDNA ends (RACE) technique, using LPS stimulated alveolar macrophages as a source of RNA.

Cattle IL-18, similar to human and rodent has been demonstrated to induce IFNγ production in synergy with IL-12 and independently of IL-12, and enhance T cell proliferation (Shoda et al., 1999). The experiments presented in this and subsequent chapters aim to: 1) evaluate the regulation of IL-18 expression in sheep, and 2) extend the knowledge of dendritic cell function.

3.2 RESULTS

3.2.1 Primer Design

Primers for the generation of ovine IL-18 were designed based on the nucleotide sequences of IL-18 from different species (human, porcine, canine, bovine). A 5' degenerate primer was designed based on a consensus sequence of rat, human, canine, porcine and murine IL-18 cDNA sequences. Sequences of the primers used in this chapter are presented in Appendix 4. Figure 3.1 shows the region of the IL-18 nucleotide sequence that were chosen for the generation of the PCR primers. Comparisons of nucleotide sequences presented in this Chapter were performed using GAP comparison software (Genetic Computer Group, Version 10 [GCG 10]; Madison, WI. USA). GAP uses the algorithm of Needleman and Wunsch (1970) to find the alignment of two sequences by maximizing the number of matches and minimizing the number of gaps. Default settings for gap weight and gap length (5.0 and 3.0 for nucleotide comparisons, 0.3 and 0.1 for amino acid comparisons, respectively) were used.

3.2.2 Cloning of Sheep IL-18

Templates, primer sets and amplification conditions utilised for all PCR reactions described in this Chapter are presented in Table 3.1. All PCR's were 'hot started' to reduce primer oligomerisation. cDNA was synthesised from 1 μg total RNA isolated from adherent alveolar cells cultured for 4 hours with 1 μg/ml LPS (*Salmonella typhimurium* lipopolysaccharide, Sigma) or 5 ng/ml PMA (Sigma) as template and oligo (dT) as primer, as described in Section 2C.2. The cDNA was then analysed by PCR utilising primers specific for the ATPase gene as a positive control for cDNA synthesis. The ATPase message should be detectable in all tissues (Shull, Schwartz and Lingrel, 1985) and any samples that were negative for this PCR product were not utilised further. In addition, cDNA synthesis reactions were set up with the RNA samples in the absence of reverse transcriptase enzyme, and 1 μl used in PCR reactions with ATPase primers.

Figure 3.1 Comparison and consensus of IL-18 sequences. Sequence alignment of IL-18 cDNA sequences using GAP software (Genetic Computer Group, Version 10 [GCG 10]; Madison, WI. USA). IL-18 sequences analysed were as follows: rat brain, AJ222813; human liver, D49950; canine macrophages, Y11133; porcine macrophages, AB010003; cattle macrophages AF124789; mouse cell line AJ002364. A dot (.) indicates gaps which were introduced to maintain alignment. A dash (-) indicates no consensus nucleotide between sequences. Sequences on which the PCR primer sets were based are shown; primer set 1 are shown in blue; primer set 2 are shown in orange; primer set 3 contains the sense primer of set 2 (orange) and antisense primer of set 4 (green); primer set 4 are shown in green, primer set 5 are shown in red.

```
          1                                                  50
Rat brain           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Human liver         GCCTGGACAG TCAGCAAGGA ATTGTCTCCC AGTGCATTTT GCCCTCCTGG
Dog macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Pig macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Cattle macrophages~~~~~~~~~ ~~~~~~~~ ~~~~~~~~~~AC AGTTCTGCTC TCCAATGCTT
Mouse cell line     ~~~~~~~~~~ ~~~~~~~~~ ~AAGCTTTAT AAAACGGGGC TGTTTCCAGG
Consensus           GCCTGGACAG TCAGCAAGGA A-G---T-AC AGTTC-G-TC TCC-TCC-GG

                    51                                                 100
Rat brain           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Human liver         CTGCCAACTC TGGCTGCTAA AGCGGCTGCC ACCTGCTGCA GTCTACACAG
Dog macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Pig macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Cattle macrophages  TCAGCGCTCC TGGCTGCCTG CTTCTGCTGC TGAAGGGGCT GCCGTCTTCT
Mouse cell line     CCACTGAGAA GTAAATCTGT AGAGCTTCTA GCAAGTGTCA TGTGACACCA
        Consensus   CCACCGA--C TGGCTGCT-- AG-GC-T--C -CAAG-GGCA G-CGACACCG

                    101                                                150
Rat brain           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Human liver         CTTCGGGAAG AGGAAAGGAA CCTCAGACCT TCCAGATCGC TTCCTCTCGC
Dog Macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Pig macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Cattle macrophages  GTAAGGAAAA GAAAGGGACC TCAAACCTTT CATATCACGT TTCCTCTCCT
Mouse cell line     TGTTGAGCCT GGGAAAGTAA AATCCTGAAT AAGATCTTAA AGAGGAAGAA
        Consensus   -TTCGGGAA- GGGAAAG-AA -CTCA----T -A-ATCTCG- TTCCTCTC--

                    151                                                200
Rat brain           ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~ATG GCTGCCATGT CAGAAGAAGG
Human liver         AACAAACTAT TTGTCGCAGG AATAAAGATG GCTGCTGAAC CAGTAGAAGA
Dog macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~ATG GCTGCTAACC TAATAGAAGA
Pig macrophages     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~ATG GCTGCTGAAC C...GGAAGA
Cattle macrophages  AAGAAGCTAT TGAGCACAGG CATAAAGATG GCTGCAGAAC AAGTAGAAGA
Mouse cell line     AGAAAACACT ATACAAAAGT TATTTTGTTA CCCCCCCAAA AATAATATAT
        Consensus   AACAAACTAT TTATCACAGG -ATAAAGATG GCTGCTGAAC -AG-AGAAGA

                    201                                                250
Rat brain           CTCTTGTGTC AACTTCAAAG AAATGATGTT TATTGACAAC ACACTTTACC
Human liver         CAATTGCATC AACTTTGTGG CAATGAAATT TATTGACAAT ACGCTTTACT
Dog macrophages     CAATTGCATC AACCTTGTGA AAATGAAATT TGTTAACAAT ACACTGTACT
Pig macrophages     CAATTGCATC AGCTTTGTGG AAATGAAGTT TATTAACAAT ACACTTTACT
Cattle macrophages  TTATTGCATC AGCTTTGTGG AAATGAAATT TATTAACAAT ACACTTTATT
Mouse cell line     TTTATATATA ATGGCTATAT TATAAAAATT ACTACAGTAT GACTGTTGTT
        Consensus   CAATTGCATC AACTTTGTGG -AATGAAATT TATTGACAAT AC-CTTTACT

                    251                                                300
Rat brain           TTATACCTGA AGATAATGGA GACTTGGAAT CAGACCACTT TGGCAGACTT
Human liver         TTATAGCTGA AGATGATGAA AACCTGGAAT CAGATTACTT TGGCAAGCTT
Dog macrophages     TTAAAGCGGA AAGTGATGAA GGCCTGGAAT CAGATTACTT TGGCAAGCTT
Pig macrophages     TTGTAGCTGA AAACGATGAA GACCTGGAAT CGGATTACTT TGGCAAGCTT
Cattle macrophages  TTGTAGCTGA AAATGATGAA GACCTGGAAT CAGATCACTT TGGCAAACTT
Mouse cell line     CAGTGGTTTT AGATAAAAAA ATATTTCATG CTGAAGTGTG CAAGAGCCTC
        Consensus   TTATAGCTGA AGATGATGAA GACCTGGAAT CAGATTACTT TGGCAAGCTT


                    301                                                350
Rat brain           CACTGTACAA CCGCAGTAAT ACGGAGCATA AATGACCAAG TTCTCTTCGT
Human liver         GAATCTAAAT TATCAGTCAT AAGAAATTTG AATGACCAAG TTCTCTTCAT
Dog macrophages     GAACCTAAAC TCTCAATCAT ACGAAATTTG AACGACCAAG TCCTCTTCGT
Pig macrophages     GAACCTAAAC TCTCAATCAT ACGAAATCTG AACGACCAAG TCCTTTTCAT
Cattle macrophages  GAACCTAAGC TCTCAATCAT ACGAAATTTG AATGACCAAG TTCTCTTCAT
Mouse cell line     AATACCTACA AGCTGCTATG AGAAAGGATG CTCTAACCAA CCAACGGCGT
        Consensus   GAATCTAAAC TCTCAGTCAT ACGAAATTTG AATGACCAAG TTCTCTTCAT

                    351                                                400
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Rat brain           TGACAAAAGA AACCCGCCTG TGTTCGAGGA CATGCCTGAT ATCGACCGAA
Human liver         TGACCAAGGA AATCGGCCTC TATTTGAAAA TATGACTGAT TCTGACTGTA
Dog macrophages     TAACGAGGGA AATCAACCTG TATTTGAGGA TATGCCCGAT TCTGACTGTA
Pig macrophages     TAACCAGGGA CATCAAGCCG TGTTTGAGGA TATGCCTGAT TCTGACTGTT
Cattle macrophages  TAACCAGGGA AATCAACCTG TCTTTGAGGA TATGCCTGAT TCTGACTGTT
Mouse cell line     TCATTTGCTT AGACTATATT CACCTGGCTT TCTGAAAGAG GCATGGT.CC
        Consensus   TAACCAGGGA AATCAACCTG TATTTGAGGA TATGCCTGAT TCTGACTGTA

                    401                                                 450
Rat brain           CAGCCAACGA ATCCCAGACC AGACTGATAA TATATATGTA CAAAGATAGT
Human liver         GAGATAATGC ACCCCGGACC ATATTTATTA TAAGTATGTA TAAAGATAGC
Dog macrophages     CAGATAATGC ACCCCATACC ATATTTATCA TCTATATGTA TAAAGATAGC
Pig macrophages     CAGATAATGC ACCTCAGACC GTATTTATTA TATATATGTA TAAAGATAGC
Cattle macrophages  CAGATAATGC ACCCCAGACC ATATTTATCA TATATATGTA TAAGGACAGC
Mouse cell line     CAGCTTTTAC AGAAGAGATG ATACTGAATA GTAACATTTA CAGTAAAGGA
        Consensus   CAGATAATGC ACCCCAGACC ATATTTATTA TAAATATGTA TAAAGATAGC

                    451                                                 500
Rat brain           GAAGTAAGAG GACTGGCTGT GACCCTATCT GTGAAGGATG GAAGGATGTC
Human liver         CAGCCTAGAG GTATGGCTGT AACTATCTCT GTGAAGTGTG AGAAAATTTC
Dog macrophages     CTCACTAGAG GTCTGGCAGT AACTATCTCT GTGAAGTATA AGACAATGTC
Pig macrophages     CTCACTAGAG GTCTGGCAGT AACCATCTCT GTGCAGTGTA AGAAAATGTC
Cattle macrophages  CTCACTAGAG GTCTGGCCGT AACCATCTCT GTGCAGTGTA AGAAAATGTC
Mouse cell line     AATGCCCGTG TTTTCTAACA TTTTTCTTCA AATTCCTTGT GCCCTGGAAA
        Consensus   CAG-CTAGAG GT-TGGCTGT AACTATCTCT GTGAAGTGTG AGAAAAT-TC

                    501                                                 550
Rat brain           TACCCTCTCC TGTAAAAACA AAATCATTTC CTTTGAG... ..........
Human liver         AACTCTCTCC TGTGAGAACA AAATTATTTC CTTTAAGGAA ATGAATCCTC
Dog macrophages     TACTCTCTCC TGTAAGAACA AAACTATTTC CTTTCAGAAA ATGAGTCCTC
Pig macrophages     TACTCTCTCC TGTAAGAACA AAACTCTTTC CTTTAAGGAA ATGAGTCCTC
Cattle macrophages  TACTCTCTCC TGTGAGAACA AAATTGTTTC CTTTAAGGAA ATGAATCCTC
Mouse cell line     ACAGGCACCA TGTGAAGGCG GGCCTGAGGC GTGCCAGGGG CAGTCTCCTC
        Consensus   AACTCTCTCC TGTGAGAACA AAATTATTTC CTTTAAGGAA ATGAATCCTC

                    551                                                 600
Rat brain           .......... .......... .......... .......... ....AAACGT
Human liver         CTGATAACAT CAAGGATACA AAAAGTGACA TCATATTCTT TCAGAGAAGT
Dog macrophages     CGGATAGTAT CAATGATGAA GGAAATGACA TCATATTCTT TCAGAGAAGT
Pig macrophages     CTGATAATAT TGATGATGAA GGAAATGACA TCATATTCTT TCAGAGAAGT
Cattle macrophages  CTGATAACAT TGATAATGAA GAAAGTGACA TCATATTCTT TCAAAGAAGT
Mouse cell line     CCTTTGCCTG CCTTCTTCCT CATTCTTGTG CCTTGGTATC ACAG.GCGCA
        Consensus   CTGATAACAT CAATGATACA G-AA-TGACA TCATATTCTT TCAGAGAAGT

                    601                                                 650
Rat brain           GTGCCAGGAC ...ACAACAA AATGGAATTT GAATCTTCCC TGTATGAAGG
Human liver         GTCCCAGGAC ATGATAATAA GATGCAATTT GAATCTTCAT CATACGAAGG
Dog macrophages     GTTCCAGGCC ATGATGATAA GATACAATTT GAGTCTTCAT TGTACAAAGG
Pig macrophages     GTTCCTGGAC ATGATGATAA GATACAGTTT GAGTCTTCAT TGTACAAAGG
Cattle macrophages  GTTCCAGGAC ATGATGATAA GATACAATTT GAGTCTTCAT TGTACAAAGG
Mouse cell line     GCTGGACCTG GTGGGGGTTC TCTGTGGTTC CATGCTTTCT GGACTCCTGC
        Consensus   GTCCCAGGAC ATGATAATAA GATGCAATTT GAATCTTCAT --TACGAAGG



                    651                                                 700
Rat brain           ACACTTTCTA GCTTGCCAAA AGGAAGATGA TGCTTTCAAA CTCGTTTTGA
Human liver         ATACTTTCTA GCTTGTGAAA AAGAGAGAGA CCTTTTTAAA CTCATTTTGA
Dog macrophages     ACACTTTCTA GCTTGTAAAA AAGAGAACGA TCTTTTCAAA CTCATTTTGA
Pig macrophages     ATACTTTCTA GCTTGTAAAA AAGAGAACGA CCTTTTCAAA CTCATTTTGA
Cattle macrophages  GTACTTTCTA GCTTGTAAAA AAGAGAATGA CCTTTTCAAA CTCATTTTGA
Mouse cell line     CTGCTGGCTT GAGCTGCTGA CAG.GCCTGA CATCTTCTGC AACCTCCAGC
        Consensus   ATACTTTCTA GCTTGT-AAA AAGAGA--GA CCTTTTCAAA CTCATTTTGA

                    701                                                 750
Rat brain           AAAGGAAGGA TGAAAATGGG GATAAATCTG TAATGTTCAC TCTTACTAAC
Human liver         AAAAAGAGGA TGAATTGGGG GATAGATCTA TAATGTTCAC TGTTCAAAAC
Dog macrophages     AAGACAAGGA TGAAAATGGG GATAAATCCA TAATGTTCAC TGTTCAAAAC
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Pig macrophages      AAGAAAAGGA TGAATGTGGA GATAAATCTA TAATGTTCAC TGTTCAAAAC
Cattle macrophages   AAAAACAGGA TGATAATAGA GATAAATCTG TAATGTTCAC TGTTCAAAAC
Mouse cell line      ATCAGGACAA AGAAAGCCGC CTCAAACCTT CCAAATCACT TCCTCTTGAC
          Consensus  AAAAAGA GGA TGAA--TGG- GATAAATCTA TAATGTTCAC TGTTCAAAAC

                     751                                                800
Rat brain            TTACATCAAA GTTAGGTATT AAGGTTTCTG TATTCCAGAA AGACGATTAG
Human liver          GAAGACTAGC TATTAAAATT TCATGCCGGG CGCAGTGGCT CACGCCTGTA
Dog macrophages      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Pig macrophages      AAGAACTAGA TATTAAAACT GCATGGTTTG AACTTTCTGG GTTTTTTTCC
Cattle macrophages   CAGAACTAGA TATTAAAATG ATATAGTTTG AA~~~~~~~~ ~~~~~~~~~~
Mouse cell line      CCAGGTAAGA TGTGTCTCTC TTTATTCGGG CACACTGGCG TGACGGTGAT
          Consensus  GAAGACTAGA TATTAATATT ----TTT-TG -ACTCT-G-A AGACGATTAG
```

Table 3.1 Sheep IL-18 cloning parameters

Template	Primers	Cycle No.	Cycle parameters	Extension	Taq enzyme details
1 µl macrophage cDNA: odT	X02813F1 X02813R1	30	94 1', 55 1', 72 1'	72 10'	1.2 units Expand high fidelity polymerase (Roche)
1 µl macrophage cDNA: odT	**Set 1** G1899241F G1899241R	35	94 1', *various* 40-55 1', 72 1'	72 10'	
1 µl macrophage cDNA: odT	**Set 2** AB010003F AB010003R	35	94 1', *various* 40-55 1', 72 1'	72 10'	
1 µl macrophage cDNA: odT	**Set 3** AB010003F ConIL-18R	35	94 1', *various* 55-60 1', 72 1'	72 10'	
1 µl macrophage cDNA: odT	**Set 4** Y11133F ConIL-18R	35	94 1', 50 1', 72 1'	72 10'	
1 µl macrophage cDNA: odT	**Set 5** AF124789F AF124789R	35	94 1', 55 1', 72 1'	72 10'	
10 ng plasmid DNA	AB010003F ConIL-18R	25	94 1', 55 1', 72 1'	72 10'	
10 ng plasmid DNA	Y11133F ConIL-18R	25	94 1', 50 1', 72 1'	72 10'	
1 µl macrophage cDNA: odT	AF004024F1 AF004024R	35	94 1', 55 1', 72 1'	72 10'	1-2 units AGS gold polymerase (Hybaid)
1 µl macrophage cDNA: odT	X54796F X54796R	35	94 0.5', 55 0.75', 72 0.5'	72 10'	
5 µl macrophage cDNA: RACE 1	RACE2 odT anchor	40	94 0.25', 55 0.5', 72 0.5'	72 7'	
1 µl RACE2 odT-anchor	RACE3 Anchor	40	94 0.25', 55 0.5', 72 0.5'	72 7'	
1 µl macrophage cDNA: odT anchor	RACE4 Anchor	'touch-down' PCR 40	(94 0.25', 60 0.25', 72 0.5') x 10 (94 0.25' 60 (-0.3/cycle), 72 30') x 30	72 7'	
0.4 µl RACE4 anchor	RACE5 Anchor	40	94 0.25', 55.5 0.5', 72 0.5'	72 7'	

Details of the PCR reaction templates, primer usage, cycling parameters and commercially available *Taq* enzyme used. Primer sequences are listed in Appendix 4.

3.2.3 RT-PCR for the IL-18 Coding Region

Primer sets 1 and 2 initially utilised various PCR cyling parameters (Table 4.1). No product was obtained in any reaction. These primers were designed from human and porcine sequences, and possibly contain regions of non-homology for the ovine IL-18 sequence. Therefore an antisense 'degenerate' primer was designed as described above, and used in conjunction with the sense primer from set 2, and a newly designed sense primer based on the canine IL-18 sequence, and designated primer set 3 and 4 respectively. To test the efficiency of primer sets 3 and 4, PCR reactions were performed using plasmid DNA containing canine IL-18 cDNA as template (Argyle et al., 1999) (10 ng). Primer set 3 resulted in no PCR product, whereas set 4 produced a product of expected size (533 bp) (not shown). Primer set 4 was utilised to amplify ovine cDNA with the condition outlined Table 3.1 and generated non-specific products. PCR reactions using primer set 4 were optimised for $MgCl_2$ concentration (not shown); 0.2 mM $MgCl_2$ was found to be optimal and used in subsequent reactions. To complement for ATPase amplification as positive control, the cDNA PCR templates were analysed for IL-1β and IL-12 p40 message using primers X54796F & X54796R, and AF004024F & AF004024R, respectively. IL-1β and IL-12 p40 gene transcription is strongly induced by LPS (Dinarello, 1998) (Trinchieri, 1998). The IL-1β-specific primers generated a product of expected size (123 bp.) using a number of cDNA templates (not shown), and these were utilised subsequent reactions. Analysis of two of these samples using primer set 4 produced multiple products ranging from 200 bp. to 800 bp. These were excised and purified using a spin-bind column (Section 2C.6). The purified products were cloned into pGEM-T-easy using TA cloning (Section 2C.7), amplified and purified using Qiagen spin columns (Section 2C.10). Positive clones were analysed by endonuclease digestion of the plasmid clones with EcoR I. The plasmids containing inserts of expected size were sequenced.

Comparison analysis using GAP software (GCG 10) showed that none of the cloned inserts displayed any significant homology to canine or human IL-18, strongly indicating that the products were not ovine IL-18 (not shown).

Primer set 5 was designed based on the bovine sequence (AF124789). These primers generated a PCR product of expected size (Figure 3.2). The product was purified using a spin-bind column and cloned as before using the TA cloning kit. Following plasmid DNA purification, the clone was sequenced across both sense and antisense strands. Analysis of this insert using GAP software revealed >98% homology with the bovine IL-18 gene. Based on sequence comparisons to other species (cattle, human, canine; Section 3.2.5), the clone was considered to represent the ovine IL-18 coding region. Subsequently, four ovine IL-18 cDNA clones were obtained from two separate RT reactions using cDNA from LPS and PMA stimulated macrophages as templates.

3.2.4 'RACE' PCR

The ovine IL-18 coding region obtained by RT-PCR using Primer set 5, which were based on bovine IL-18, incorporated the initiation and stop codons. It was necessary to sequence through these regions to obtain the primary sheep sequence, so the rapid amplification of cDNA ends (RACE) technique was employed. This was achieved using the 5'3' RACE system (Roche Molecular Biochemicals) (Section 2C.3). A schematic representation of the RACE technique is shown in Figure 3.3. Manufacturers control primers were used to analyse the cDNA synthesis and 5' polyA-tailing reactions (not shown), samples that produced positive results were utilised further. RNA samples were analysed by RT-PCR utilising ATPase primers (as previously described), and Primer set 5 to demonstrate the presence of IL-18 message (not shown). Positive samples were utilised in the RACE strategy.

Both the 3' and 5' end amplification utilised nested primer PCR strategies. Figure 3.4 shows the results of primary and secondary PCR for the 3' region of ovine IL-18. The first round of PCR used 1 μl of cDNA as template, the primers anchor and RACE4 and a 'touchdown' PCR strategy (Don et al., 1991) to increase the abundance of specific product. A nested PCR was carried out using 0.4 μl of this DNA as template. Agarose gel electrophoresis analysis of the nested PCR reactions utilising primers anchor and RACE5 are shown in Figure 3.4b. Multiple bands were

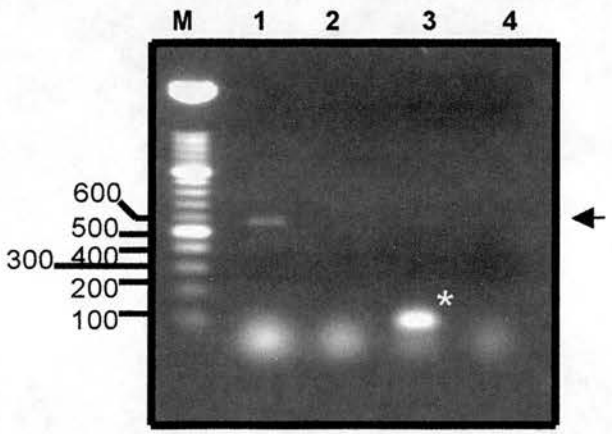


Figure 3.2 PCR amplification of IL-18 cDNA from LPS stimulated macrophage RNA.

M = Marker DNA

Lane 1: PCR product using primer set 5 (for details see Table 3.1). The single product of 609 bp. (indicated by the arrow), was purified, cloned and sequenced and concluded to be ovine IL-18 based on strong homolgy with published IL-18 sequences.

Lane 2: negative control (template DNA omitted) for reaction utilising primer set 5.

Lane 3: ATPase internal control using primers X02813F & X02813R1 (Table 3.1), produced a product of the expected size; 118 bp. (indicated with an asterix)

Lane 4: negative control (template DNA omitted) for reaction utilising ATPase-specific primers.

Figure 3.3 Amplification of the 5'/3' ends of sheep IL-18 cDNA using the Roche RACE system

a) Specific primers were designed based on the consensus nucleotide sequence of the ovine IL-18 coding region. The positions of the RACE primers are shown. Primers are shown in blue. RT-PCR reactions were performed using stimulated macrophage RNA as template.

b) Amplification of the 5' region was achieved by synthesising cDNA utilising the specific primer RACE 1. This product was purified using a spin-bind column, dA-tailed, and was used as template for a primary PCR reaction utilising primers oligo(dT)-anchor and RACE2. A nested PCR strategy was then utilised using the primary PCR product as template and primers anchor and RACE3.

c) The 3' region was amplified by performing a cDNA synthesis reaction using the oligo(dT)-anchor primer. This product was used as template for a primary PCR reaction utilising primers RACE4 and anchor. Nested PCR was performed on a purified fragment of expected size (~400bp.), utilising the RACE5 and anchor primers.

The diagram is not to scale.

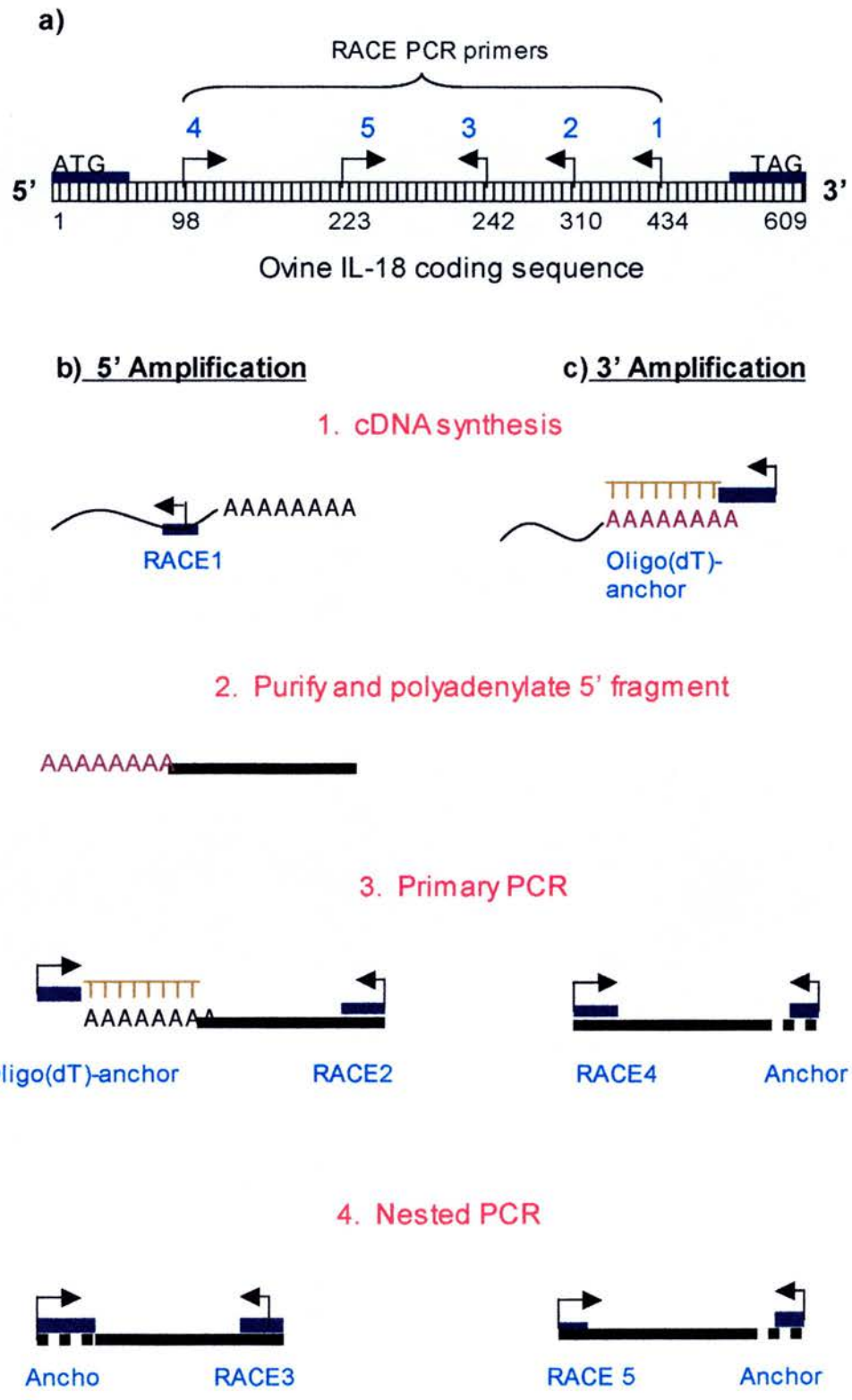

a)
RACE PCR primers
4
5
3
2
1
ATG
TAG
5'
3'
1
98
223
242
310
434
609
Ovine IL-18 coding sequence
b) 5' Amplification
c) 3' Amplification
1. cDNA synthesis
AAAAAAAA
RACE1
AAAAAAAA
Oligo(dT)-anchor
2. Purify and polyadenylate 5' fragment
AAAAAAAA
3. Primary PCR
AAAAAAAA
Oligo(dT)-anchor
RACE2
RACE4
Anchor
4. Nested PCR
Anchor
RACE3
RACE 5
Anchor

Figure 3.4 Amplification and cloning of the 3' region of sheep IL-18 cDNA

M: marker DNA. -ve: template omitted as negative control

a) PCR using macrophage cDNA as template

1, 2 and 3: individual PCR reactions using primers oligo(dT)-anchor and RACE4 (See Table 3.1 for details)

b) Nested PCR utilising DNA generated with primers oligo(dT)-anchor and RACE4 (Figure 3.4a) as template

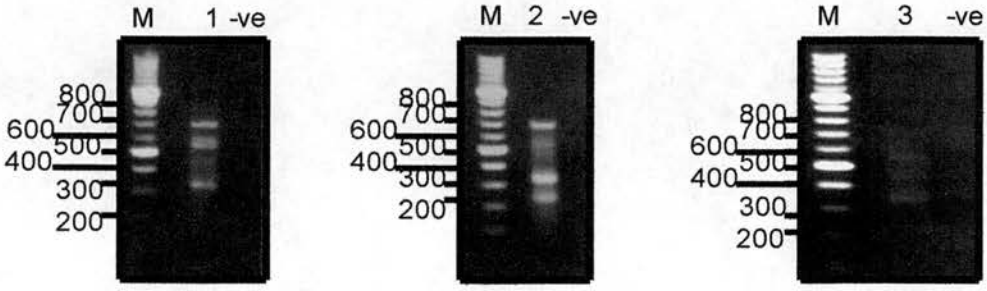
1, 2 and 3: individual PCR reactions utilising corresponding DNA template samples, using primers anchor and RACE5

Band of expected size (401 bp.) is indicated with an arrow.

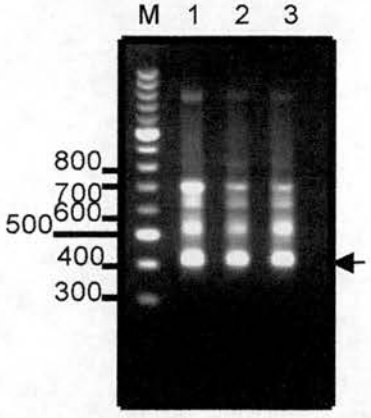
c) Cloning of the 400 bp. product in Figure 3.4b, which is indicated by the arrow: EcoR I digest of one positive clone per individual amplification reaction (1, 2 and 3).

Amplification parameters are shown in Table 3.1

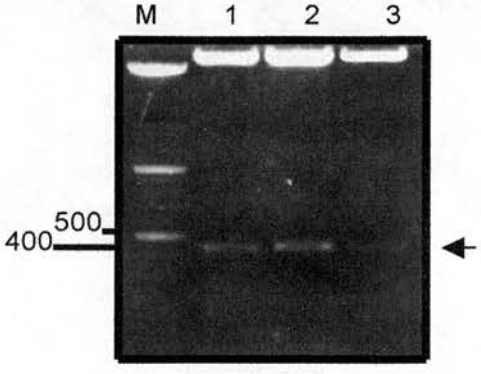
a) Primary PCR reactions



b) Nested PCR reactions



c) Cloning of the ~400 bp. product



present in all reactions, the size of the PCR product of interest based on the full cDNA sequence of bovine IL-18, was the strongest product of approximately 400 bp. This was excised, purified and cloned as previously described. Restriction digests of purified plasmid DNA for the three clones are shown (Fig. 3.4c). For the 5' ends, 1 μg of total RNA was reverse transcribed using the specific primer RACE1 and the dA-tailed cDNA was amplified for two rounds of PCR using the specific primers RACE2 and RACE3 with the oligo(dT)-anchor and anchor primer respectively. Templates for the reactions were 5 μl of cDNA for the primary PCR and 1 μl of that reaction for the nested PCR reaction. The results are shown in Figure 3.5. The nested PCR reaction produced a single product of expected size that was purified with a spin-bind column, purified and cloned as above. Sequencing and analysis of the 3' and 5' RACE clones confirmed these were sheep IL-18 (discussed below).

3.2.5 Sequence Analysis

The nucleotide sequence of three overlapping cDNA fragments, representing the coding region of IL-18, 5' and 3' RACE, each from at least two independent RNA samples and multiple secondary PCR reactions, were analysed using GCG 10. Consensus sequence was deduced using the PILEUP programme to compare multiple overlapping clones. PILEUP creates a multiple sequence alignment using progressive pairwise alignments. Figure 3.6 shows an alignment of representative clones containing the 3' region, coding region and 5' region of ovine IL-18. Where nucleotide ambiguities existed, three out of four identical residues were taken as the consensus sequence.

Figure 3.7 shows the nucleotide sequence of sheep IL-18 and predicted amino acid composition of the protein. Sheep IL-18 has a coding sequence of 582 base pairs, including stop codon, encoding a precursor peptide length of 193 amino acids. The nucleotide sequence has been submitted to EMBL/GenBank Data Libraries under accession number AJ401033.

Production of bioactive human IL-18 is associated with cleavage of the full-length precursor protein (proIL-18) between Asp_{36} and Try_{37} by caspase-1 (also known as IL-1β converting enzyme [ICE]) (Ghayur et al., 1997). Based on

Figure 3.5 Amplification of the 5' region of sheep IL-18 cDNA

a) PCR using macrophage polyA-cDNA produced with primer RACE1 as template

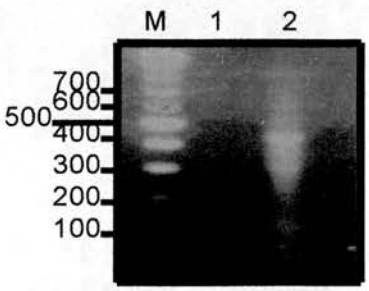
M: marker DNA, 1: negative control, 2: PCR product using primers oligo(dT)-anchor and RACE2

b) Nested PCR utilising DNA generated with primers oligo(dT)-anchor and RACE2 (Figure 4.5a) as template

M: marker DNA, 1: PCR product using primers anchor and RACE3, 2: negative control

Amplification parameters are shown in Table 3.1

a)



b)

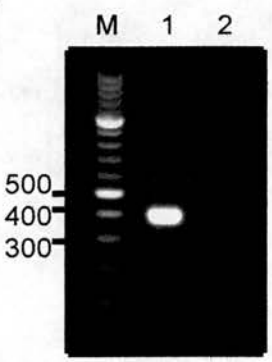


Figure 3.6 Alignment of cDNA fragments representing the coding, 5' and 3' regions of sheep IL-18. The overlapping fragments were aligned using the PILEUP programme of GCG 10. Internal clones containing the start and stop codons are indicated in red (Clone 1 & 2), 5' clones in purple (Clone 3 & 4), and 3' clones in green (Clone 5 & 6). The positions of the PCR primers used to obtain the IL-18 coding region are shown; sense primers in blue, antisense in red.

Figure 3.6

```
          1                                                  50
Clone3    CTCTAGTCGG ATCCGGACTG GCAGAGAGTT CTGCTTTCCA GTGCTTTCAG
Clone4    ~TCTAGTCGG ATCCGGACTG GCAGAGAGTT CTGCTTTCCA GTGCTTTCAG
Clone1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone2    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone5    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          51                                                 100
Clone3    CTCTCCTGGG GGTCTGCTTC TGCTGCTGAA GGGGCTGCCG TCTTCTATAA
Clone4    CTCTCCTGGC GGTCTGCTTC TGCTGCTGAA GGGGCTGCCG TCTTCTATAA
Clone1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone2    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone5    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          101                                                150
Clone3    GGAAAAGAAA AGGACCTCAA ACCGTTCAGA TCACGTTTCC TCTCCTAGGA
Clone4    GGAAAAGAAA AGGACCTCAA ACCGTTCAGA TCACGTTTCC TCTCCTAGGA
Clone1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone2    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone5    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          151                                                200
Clone3    AGCTATTGAG CACAGGCATA AAGATGGCTG CAGAACCAGT AGAAGACAAT
Clone4    AGCTATTGAG CACAGGCATA AAGATGGCTG CAGAACCAGT AGAAGACAAT
Clone1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~ATGGCTG CAGAACAAGT AGAAGACAAT
Clone2    ~~~~~~~~~~ ~~~~~~~~~~ ~~~ATGGCTG CAGAACAAGT AGAAGACAAT
Clone5    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          201                                                250
Clone3    TGCATCAGCT TTGTGGAAAT GAAATTTATT AACAATACAC TTTATTTTGT
Clone4    TGCATCAGCT TTGTGGAAAT GAAATTTATT AACAATACAC TTTATTTTGT
Clone1    TGCATCAGCT TTGTGGAAAT GAAATTTATT AACAATACAC TTTATTTTGT
Clone2    TGCATCAGCT TTGTGGAAAT GAAATTTATT AACAATACAC TTTATTTTGT
Clone5    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          251                                                300
Clone3    AGCTGAAAAT GGC...GACC TGGAATCAGA TCACTTTGGC AAGCTTGAAC
Clone4    AGCTGAAAAT GGCGAAGACC TGGAATCAGA TCACTTTGGC AAGCTTGAAC
Clone1    ~~~~~~~~~G GCGAAGGACC TGGAATCAGA TCACTTTGGC AAGCTTGAAC
Clone2    AGCTGAAAAT GGCGAAGACC TGGAATCAGA TCACTTTGGC AAGCTTGAAC
Clone5    ~~~~~~~~~~ ~~~~~~~~~~ ~GGAATCAGA TCACTTTGGC AAGCTTGAAC
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          301                                                350
Clone3    CTAAGCTCTC AATCATACGA AATTTGAACG ACCGAGTTCT CTTCATTAGC
Clone4    CTAAGCTCTC AATCATACGA AATTTGAACG ACCAAGTTCT CTTCATTAGC
Clone1    CTAAGCTCTC AATCATAGGA AATTTGAACG ACCAAGTTCT CTTCATTAGC
Clone2    CTAAGCTCTC AATCATACGA AATTTGAACG ACCAAGTTCT CTTCATTAGC
Clone5    CTAAGCTCTC AATCATACGA AATTTGAACG ACCAAGTTCT CTTCATTAGC
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

```
          351                                                        400
Clone3    CAGGGAAATC AACCTGTCTT TGAGGATATG CCTGATTCTG ACTGTTCAGA
Clone4    CAGGGAAATC AACCTGTCTT TGAGGATATG CCTGATTCTG ACTGTTCAGA
Clone1    CAGGGAAATC AACCTGTCTT TGAGGATATG CCTGATTCTG ACTGTTCAGA
Clone2    CAGGGAAATC AACCTGTCTT TGAGGATATG CCTGATTCTG ACTGTTCAGA
Clone5    CAGGGAAATC AACCTGTCTT TGAGGATATG CCTGATTCTG ACTGTTCAGA
Clone6    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~TCAGA

          401                                                        450
Clone3    TAATGCACCC CAGACC~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone4    TAATGCACCC CAGACC~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone1    TAATGCACCC CAGACCATaT TTATcAtATA TATGTATAAG GACAGCCTCA
Clone2    TAATGCACCC CAGACCATAT TTATCATATA TATGTATAAG GACAGCCTCA
Clone5    TAATGCACCC CAGACCATAT TTATCATATA TATGTATAAG GACAGCCTCA
Clone6    TAATGCACCC CAGACCATAT TTATCATATA TATGTATAAG GACAGCCTCA

          451                                                        500
Clone3    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone4    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone1    CTAGAGGTCT GGCTGTAACC ATCTCTGTGC AGTGTAAGAA AATGTCTAC
Clone2    CTAGAGGTCT GGCTGTAACC ATCTCTGTGC AGTGTAAGAA AATGTCTAC
Clone5    CTAGAGGTCT GGCTGTAACC ATCTCTGTGC AGTGTAAGAA AATGTCTAC
Clone6    CTAGAGGTCT GGCTGTAACC ATCTCTGTGC AGTGTAAGAA AATGTCTAC

          501                                                        550
Clone3    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone4    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone1    TCTCTCCTGT GAGAACAaAA TTATTTCCTT TAAGGAAATG AATCCTCCTG
Clone2    TCTCTCCTGT GAGAACAAAA TTATTTCCTT TAAGGAAATG AATCCTCCTG
Clone5    TCTCTCCTGT GAGAACAAAA TTATTTCCTT TAAGGAAATG AATCCTCCTG
Clone6    TCTCTCCTGT GAGAACAAAA TTATTTaCTT TAAGGAAATG AATCCTCCTG

          551                                                        600
Clone3    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone4    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone1    ATAACATTGA TAATGAAGGA AGTGACATCA TATTCTTTCA GAGAAGTGTT
Clone2    ATAACATTGA TAATGAAGGA AGTGACATCA TATTCTTTCA GAGAAGTGTC
Clone5    ATAACATTGA TAATGAAGGA AGTGACATCA TATTCTTTCA GAGAAGTGTT
Clone6    ATAACATTGA TAATGAAGGA AGTGACATCA TATTCTTTCA GAGAAGTGTT

          601                                                        650
Clone3    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone4    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone1    CCAGGACATG ATGATAAGAT ACAATTTGAG TCTTCATTGT ACAAAGGGTA
Clone2    CCAGGACATG ATGATAAGAT ACAATTTGAG TCTTCATTGT ACAAAGGGTA
Clone5    CCAGGACATG ATGATAAGAT ACAATTTGAG TCTTCATTGT ACAAAGGGTA
Clone6    CCAGGACATG ATGATAAGAT ACAATTTGAG TCTTCATTGT ACAAAGGGTA

          651                                                        700
Clone3    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone4    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone1    CTTTCTAGCT TGTAAAAAAG AGAATGACCT TTTCAAACTC ATTTTGAAAA
Clone2    CTTTCTAGCT TGTAAAAAAG AGAATGACCT TTTCAAACTC ATTTTGAAAA
Clone5    CTTTCTAGCT TGTAAAAAAG AGAATGACCT TTTCAAACTC ATTTTGAAAA
Clone6    CTTTCTAGCT TGTAAAAAAG AGAATGACCT TTTCAAACTC ATTTTGAAAA
```

```
          701                                                       750
Clone3    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone4    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Clone1    GACAGGATGA TAATAGAGAT AAATCTATAA TGTTCACTGT TCAAAACAAG
Clone2    GACAGGATGA TAATAGAGAT AAATCTATAA TGTTCACTGT TCAAAACAAG
Clone5    GACAGGATGA TAATAGAGAT AAATCTATAA TGTTCACTGT TCAAAACCAG
Clone6    GACAGGGTGA TAATAGAGAT AAATCTATAA TGTTTACTGT TCAAAACCAG

          751                    776
Clone3    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~
Clone4    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~
Clone1    AACTAG~~~~ ~~~~~~~~~~ ~~~~~~
Clone2    ~~~~~~~~~~ ~~~~~~~~~
Clone5    AACTAGAAAT TAAAATGGCA TAGTTT
Clone6    AACTAGAAAT TAAAATGGCA TAGTTT
```

Figure 3.7 Nucleotide and predicted amino acid sequence for sheep IL-18 unprocessed polypeptide. The nucleotide sequence shown in upper case letters represents the coding region of 582 bp. encoding a precursor protein of 193 amino acids. Start codon is underlined and the stop codon is marked with an asterisk. The predicted caspase-1 (ICE) cleavage site is written in orange, the arrow indicates the amino acid terminus of the mature cleavage site 3' to the N-terminal leader sequence. The blue letters represent the IL-1 signature sequence, F-(X_{12})-FES-(X_6)-FL.

```
tctagtcggatccggactggcagagagttctgctttccagtgctttcagctctcctgg
cggtctgcttctgctgctgaaggggctgccgtcttctataaggaaaagaaaaggacct

                                                          1
caaaccgttcagatcacgtttcctctcctaggaagctattgagcacaggcataaagATG
                                                          M

                                        10
GCT GCA GAA CCA GTA GAA GAC AAT TGC ATC AGC TTT GTG GAA ATG
 A   A   E   P   V   E   D   N   C   I   S   F   V   E   M
             20                                      30
AAA TTT ATT AAC AAT ACA CTT TAT TTT GTA GCT GAA AAT GGC GAA
 K   F   I   N   N   T   L   Y   F   V   A   E   N   C   A
                                    40
GAC CTG GAA GGC GAA TCA GAT CAC TTT GGC AAG CTT GAA CCT AAG
 G   E   D   L   E   S   D   H   F   G   K   L   E   P   K
             50                                      60
CTC TCA ATC ATA CGA AAT TTG AAC GAC CAA GTT CTC TTC ATT AGC
 L   S   I   I   R   N   L   N   D   Q   V   L   F   I   S
                                    70
CAG GGA AAT CAA CCT GTC TTT GAG GAT ATG CCT GAT TCT GAC TGT
 Q   G   N   Q   P   V   F   E   D   M   P   D   S   D   C
             80                                      90
TCA GAT AAT GCA CCC CAG ACC ATA TTT ATC ATA TAT ATG TAT AAG
 S   D   N   A   P   Q   T   I   F   I   I   Y   M   Y   K
                                   100
GAC AGC CTC ACT AGA GGT CTG GCT GTA ACC ATC TCT GTG CAG TGT
 D   S   L   T   R   G   L   A   V   T   I   S   V   Q   C
            110                                     120
AAG AAA ATG TCT ACT CTC TCC TGT GAG AAC AAA ATT ATT TCC TTT
 K   K   M   S   T   L   S   C   E   N   K   I   I   S   F
                                   130
AAG GAA ATG AAT CCT CCT GAT AAC ATT GAT AAT GAA GGA AGT GAC
 K   E   M   N   P   P   D   N   I   D   N   E   G   S   D
            140                                     150
ATC ATA TTC TTT CAG AGA AGT GTT CCA GGA CAT GAT GAT AAG ATA
 I   I   F   F   Q   R   S   V   P   G   H   D   D   K   I
                                   160
CAA TTT GAG TCT TCA TTG TAC AAA GGG TAC TTT CTA GCT TGT AAA
 Q   F   E   S   S   L   Y   K   G   Y   F   L   A   C   K
            170                                     180
AAA GAG AAT GAC CTT TTC AAA CTC ATT TTG AAA AGA CAG GAT GAT
 K   E   N   D   L   F   K   L   I   L   K   R   Q   D   D
                                   190
AAT AGA GAT AAA TCT ATA ATG TTC ACT GTT CAA AAC AAG AAC TAG
 N   R   D   K   S   I   M   F   T   V   Q   N   K   N   *

aaattaaaatggcatagttt
```

comparisons with human IL-18, the predicted ovine IL-18 peptide has a leader sequence of 36 amino acids terminating in Asp. When cleaved, ovine IL-18 would yield a mature protein of 157 amino acids, with a molecular weight of 18 KDa, as predicted by the The ExPASy (Expert Protein Analysis System) package (Swiss institute of Bioinformatics; http://ca.expasy.org/tools/pi_tool.html).

Analysis using the Seqsee v1.1 (University of Alberta, Canada; www.pence.ualberta.ca/ftp/seqsee/index.html) package has predicted that the ovine IL-18 amino acid sequence, like IL-18 from all other species investigated, contains the IL-1 signature sequence, F-(X_{12})-FES-(X_6)-FL, and no predicted hydrophobic sequence at position nt. 138 - 161. In addition, the predicted mature IL-18 peptide of sheep contains four cysteine residues, and like all other species but cattle (Shoda et al., 1999), it possesses no N-glycosylation site (N-X-S/T).

Ovine IL-18 was compared at the nucleotide and amino acid sequence level to other known IL-18 sequences using the GAP programme of GCG 10. Figure 3.8 shows the alignment of the predicted amino acid sequence of sheep, cattle, porcine, human and mouse IL-18, the ovine sequence differs to other IL-18 amino acid sequences at two positions; Gly_{30} and Ser_{59}. Table 3.2 shows the percentage identity at both nucleotide and amino acid level of unprocessed ovine IL-18 sequence to the equivalent sequences in other species. A high degree of identity (97% - 71%) is seen between all mammalian IL-18 sequences; but sheep IL-18 displays only 42% identity with chicken IL-18 (Table 3.2).

```
ovine   MAAEPVEDNC ISFVEMKFIN NTLYFVAENG EDLESDHFGK LEPKLSIIRN
cattle  ....Q...Y. .......... .........D E......... ..........
porcine.....-.... .......... .........D E.....Y... ..........
human   .......... .N..A....D .....I...D E.....Y... ..S...V...
mouse   ...MS-..S. VN.K.....D .....IP.EN G.....N..R LHCTTAV...

ovine   LNDQVLFISQ GNQPVFEDMP DSDCSDNAPQ TIFIIYMYKD SLTRGLAVTI
cattle  ........N. .......... .......... .......... ..........
porcine........N. .H.A...... .......... .V......... ..........
human   ........D. ..R.L....T ....R....R .....S.... .QP..M....
mouse   I......VDK -R.....D.T .I.Q.ASD.. .RL........ .EV......L

ovine   SVQCKKMSTL SCENKIISFK EMNPPDNIDN EGSDIIFFQR SVPGHDDKIQ
cattle  .......... ......V... .......... .E......... ..........
porcine.......... ..K.KTL... ..S......D ..N........ ..........
human   ..K.E.I... .......... ........KD TK........ ......N.M.
mouse   ..KDS..... ..K......E ..D..E...D IQ..L....K R....-N.ME

ovine   FESSLYKGYF LACKKENDLF KLILKRQDDN RDKSIMFTVQ NKN*
cattle  .......... .......... .......... ....V..... .Q.*
porcine.......... .......... .....EK.EC G......... ...*
human   ....S.E... ...E..R... .....KE.EL G.R....... .ED*
mouse   ......E.H. ...Q..D.A. .....KK.E. G...V...LT .LHQS*
```

Figure 3.8 Alignment of the predicted un-processed IL-18 amino acid sequences derived from ovine, cattle (Shoda et al., 1999), porcine (Muneta et al., 1999), human (Ushio et al., 1996), and mouse (Okamura et al., 1995b) mRNA samples. Non-consensus amino acids are indicated, a dot (.) represents consensus to the sheep amino acid sequence, an asterix (*) represents a stop codon.

Table 3.2

	Nucleotide	Amino acid
Cattle AF124789	96	97
Porcine AB010003	92	93
Equine Y11131	91	87
Canine Y11133	89	85
Human D49950	82	83
Rat U77776	71	71
Mouse D49949	70	73
Chicken AJ277865	40	42

Comparison of the percentage identity at the nucleotide and amino acid level between ovine IL-18 and other IL-18 sequences. The species and Genbank accession number of the individual sequences is shown. These results were obtained using the GAP programme of GCG 10.

3.3 DISCUSSION

The inability of primer sets 1 – 4 to detect IL-18 cDNA in the stimulated macrophage samples could have arisen for a variety of reasons. The Tm°C of the sense and antisense primers of sets 1 and 2 were not optimally matched, and although various annealing temperatures were used, simultaneous binding of the primers to target DNA may not have occurred. In addition, subsequent analysis of identity of the primer sets to the sheep IL-18 sequence derived in this study revealed portions of ambiguity in both the sense and antisense primer of set 1, and all possible versions of the degenerate antisense primer (sets 3 and 4) (not shown). Together, it is likely that the problem was associated with the inability of the primers to bind to their 'target' under the conditions used in the PCR reactions described in this section. The primers based on the sequence of cattle IL-18 (primer set 5), resulted in the amplification of sheep IL-18 cDNA. Indeed, the sheep pro-IL-18 cDNA nucleotide sequence displays 97% homology with the cattle sequence, nucleotide anomalies between the sequences were not confined to a particular region indicating a general conservation of IL-18 sequence between these species.

The RACE technique employed to obtain the primary sheep sequence utilised specific primers and an adapted primer at the 3' and 5' ends of the molecule. The synthesis of cDNA through the start codon utilised a specific primer, and amplification of this product using nested PCR produced a single product. In contrast, amplification of the 3' cDNA end resulted in non-specific product in both the primary and secondary PCR reactions. cDNA synthesis in the 3' RACE reaction utilised an oligo(dT)-adapter primer. The appearance of non-specific product is generally due to mispriming of one or both of the PCR primers, and these products will be amplified over increasing number of cycles, particularly where target template is present only in small amounts. An absolute requirement for two rounds of PCR amplification to obtain detectable levels of IL-18 RACE products indicated that the target cDNA was indeed at low levels. The [$MgCl_2$] and annealing temperature were adjusted, which resulted in a loss of products (not shown). It appeared that the presence of spurious bands following amplification of the 3' region was due to a lack of specificity of the antisense primer as the sense primers used in

the nested PCR reactions displayed specificity for 100% homology to the sheep IL-18 sequence (analysed using BLAST software www.hgmp.mrc.ac.uk). Thus, the touchdown PCR strategy was employed for the primary PCR reaction of the 3' ends, and IL-18 cDNA was isolated.

The peptide sequence of ovine IL-18, deduced from nucleotide sequence has a high degree of identity to that of IL-18 of cattle, porcine and horse, and >70% identity to human and mouse. The absence of a conventional signal peptide in proIL-18 isolated from mice and humans (Ushio et al., 1996; Okamura et al., 1995b) makes it probable that this peptide is not secreted by cells. Ghayur et al. (1997) generated a mutant proIL-18 peptide with a single amino acid substitution at the caspase-1 cleavage site (Asp_{35} – Asn_{36}) substituting the aspartic acid reside with an alanine (Ghayur et al., 1997). Following *in vitro* synthesis, and incubation with caspase-1, these mutant proteins failed to exhibit biological activity (measured by IFNγ production by mitogen-activated splenocytes) (Ghayur et al., 1997). These results demonstrated a requirement for caspase-1 activity in IL-18 activity. In support of this, Gu et al. (1997) showed that approximately 10% of the mature IL-18 was exported from COS cells transfected with an expression plasmid for proIL-18 in combination with one encoding wild-type ICE (caspase-1), whereas less than 1% of pro IL-18 was exported when cells were transfected with proIL-18 plasmid DNA alone (Gu et al., 1997). In agreement with this finding, it has been shown that murine Kupffer cells isolated from *P. acnes* challenged mice produce a mature active form of IL-18 intracellularly and also secrete the mature IL-18 into the culture medium after LPS stimulation (Tsutsui et al., 1997). These results demonstrate that caspase-1 cleavage of proIL-18 is required for biological activity, and that mature IL-18 is secreted from the cells as a mature functional protein, although the mechanism of secretion is not yet known. As with human and murine IL-18 (Okamura et al., 1995b; Ushio et al., 1996), the sheep amino acid sequence of the precursor proteins appears to lack a conventional signalling sequence with no hydrophobic sites. Sheep pro IL-18 contains a putative caspase-1 cleavage site at positions 36-37 (Figure 3.6), suggesting that cellular handling of sheep IL-18 is similar to that for mouse and human.

The production of mouse and human IL-18 is reported to be primarily attributed to cells of the monocyte-macrophage cell lineage (Okamura et al., 1995b; Kohno et al., 1997). The amplification of sheep IL-18 cDNA from LPS stimulated alveolar macrophages suggests a similarity in this species. Constitutive expression for this peptide has been reported for human MoDCs (Gardella et al., 1999; Stoll et al., 1998) murine DCs isolated from various tissues (Stoll et al., 1998), PBMC (Marshall et al., 1999; Puren, Fantuzzi and Dinarello, 1999), and murine splenocytes (Puren, Fantuzzi and Dinarello, 1999). To investigate these observations in sheep lymph DCs, IL-18 was included in an RNase protection assay, the development of which is described in the following Chapter.

Chapter 4: The RNase Protection Assay: A Method For Evaluating Ovine Cytokine Expression.

4.1 INTRODUCTION

Analysis of cytokine production has been somewhat limited in studies of ruminants compared with those of rodents and humans. This is because of the limitations of available reagents. Analysis of cytokine production is performed by a number of methods, including radioimmunoassays (RIA), enzyme-linked immunosassays (ELISA), cytometric bead assays or intracellular cytokine staining and flow cytometry. These methods rely on the detection of proteins by mAb and to date few ovine-cytokine-specifc mAb's are available. The development of assays for sheep cytokines are also few; to date ELISAs have been developed for sheep IL-1β, IL-6, IL-8, IL-12, IFNγ, GM-CSF and TNFα (Eagan et al., 1994; Emery and Davey, 1995; Entrican et al., 1996; Haig et al., 1996, Hope et al., 2002, McWaters et al., 2000; Rothel et al., 1997).

The next best opportunity to study a wide array of cytokine responses is the evaluation of cytokine gene expression. The limitations of this are clear, in that detection of mRNA does not necessary correlate with secreted protein. However, many cytokine mRNA species are relatively short lived (Carter and Malter, 1991), and not stored as accumulated cytokine proteins. This provides an important control mechanism regulating the release of biologically potent mediators. The demonstration of biological activity or the direct evaluations of cytokine secretion, seem to correlate with the expression kinetics of certain cytokines such as IFNγ, TNFα and IL-1β (Entrican et al., 1992; Becker, Devlin and Haskill, 1989; Nash et al., 1992). Methods of mRNA measurement include Northern blotting, quantitative PCR methods such as competitive (QC-) RT-PCR and real-time PCR, and the RNase protection assay.

RT-PCR is a specific, highly sensitive method of mRNA transcript detection, and theoretically can detect transcripts of one copy from the original mRNA mix. The limiting factor in RT-PCR is the production of cDNA from RNA; incomplete transcription missing the 3' end of mRNA molecules can account for wrong negative results. Differences in the kinetics of each amplification; primer annealing parameters, and sample unfolding and replication, mean that standard RT-PCR cannot be used as a quantitative assay. Quantitative real-time PCR is becoming more widely used, and permits direct quantification of mRNA transcripts in the starting material. Amplification efficiencies are compared to standards, thus allowing a calculation on the relative amount of starting material. To be comfortable that differences in amplification kinetics are not compounded during amplification reactions, multiple controls and samples are preferable. Approximately four transcripts can be assessed by real-time PCR at one time. The materials required to perform real-time PCR are rather expensive and this must be taken into account when considering this technique.

Direct detection of RNA species by specific anti-sense labelled probes using the Northern blotting technique is advantageous for identifying transcripts of unknown size, and for analysing topological features of mRNA transcripts. Degradation of the 3' and 5' ends of mRNA molecules is commonly a problem in Northern blotting. Probe hybridisation is performed in a one-dimensional gel matrix, therefore stringent hybridisation relies on homologous binding of full-length transcripts by specific probes. In addition, background readings are strong, so the sensitivity of this assay can be compromised.

The RNase protection assay (Section 4.1.2) negates some of the disadvantages experienced with Northern blotting. Hybridisation of antisense probes is performed in solution, resulting in stronger binding compared to targets within gel matrices. High quality of RNA is not as crucial in the RPA compared to Northerns because short segments (100 – 400 nt.) of the mRNA transcripts are targeted. Because of these factors, the RNase protection assay is considerably more sensitive than Northerns. In addition, a major advantage of the RPA over northern blotting and QC-PCR is that multiple transcripts can be evaluated in one sample.

4.1.2 The RNase Protection Assay

The RNase protection assay involves the hybridisation of cellular RNA to complementary radiolabelled RNA probes (riboprobes) of known size. Un-hybridised probes and RNA are removed using single-strand specific RNase's, such as RNase A and RNase T1. The RNase enzymes are then inactivated and the purified double stranded RNA species, so called 'protected' molecules analysed by PAGE, phosphorimaging and densitometry. This technique was first developed by Melton and colleagues (1984) and was used as an alternative to nuclease S1 protection assays designed for mapping mRNA topological features, such as intron-exon borders and sites of transcription initiation and termination. When a molar excess of riboprobe is used, the amount of protected molecule is directly proportional to the abundance of the transcript in the RNA sample. Specific transcripts can be identified by size, and the quantity ascertained by normalisation to a house-keeping gene. Because of the use of radiolabelled probes, the RNase protection assay is extremely specific and highly sensitive, and provides a valuable tool for the quantification of mRNA transcripts.

4.1.3 Chapter Aims

In collaboration with Dr. Anton Gossner, an RNase protection assay (RPA) was developed specifically for eleven sheep cytokines (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, GM-CSF, IFNγ, TNFα and TGFβ), and two house-keeping genes (GAPDH and ATPase). The aims of this chapter were to standardise the RPA, and determine the quantitative nature and sensitivity thresholds of the assay. These were achieved by analysing the abundance of specific transcripts in titrated samples of RNA, and the use of plasmid expressed positive control RNA. Validation of the RPA was undertaken by analysing the expression of sheep alveolar macrophages in response to stimulation *in vitro* with LPS. Alveolar macrophages originate from precursors in the bone marrow and blood (van Furth, Raeburn and van Zwet, 1979). The same blood precursor has a bi-potenital for macrophage and DC development (Reid et al., 1992; Caux et al., 1996), and macrophages and DCs share many properties. In particular, kinetics of cytokine production and expression of surface

molecules are similar for macrophages and DCs in response to innate signals such as components of microorganisms including LPS (Ammon et al., 2000; Brodskyn et al., 2002). The macrophage therefore, is an ideal candidate for the standardisation of the RPA for the analysis of DC cytokine expression. DCs are a trace population *in vivo*, whereas relatively large numbers of alveolar macrophages can be easily accessed by bronchoalveolar lavage. The data generated from these experiments were used to test the assumption that LPS induces the expression of cytokines in AM that are involved in the innate inflammatory response.

4.2. RESULTS

4.2.1 Establishment of the RNase Protection Assay (RPA)

4.2.1.1 Construction of Riboprobe Templates

RPAs depend on the hybridisation of target mRNAs with specific complementary radiolabelled riboprobes. Riboprobes were generated *in vitro* from cDNA sub-clones representing sequences in mature mRNA of various sheep cytokines. When designing the cDNA clones, a number of factors were taken into consideration:

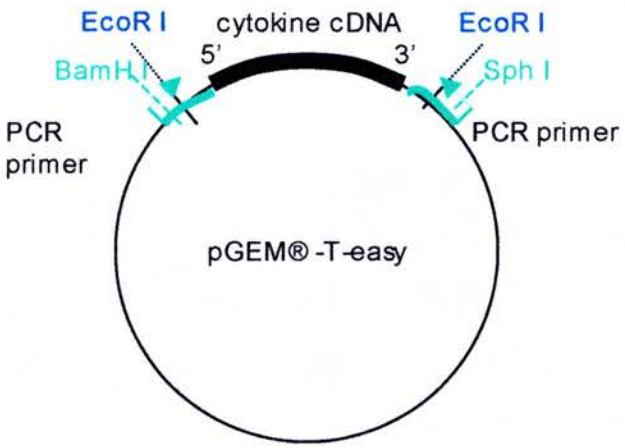
- So that the hybridised probes could be clarified on a sequencing gel, the cytokine cDNA fragments were selected to have sizes ranging from 100 – 350 bp. To allow resolution, the probes differed by at least 15 nucleotides (nt).
- To avoid non-specific hybridisation, the probes could not contain significant homology for other known ovine complementary DNAs. As such, the chosen sequences were screened using BLAST software (www.hgmp.mrc.ac.uk) and those displaying less than 5% homology to other known mammalian cDNA sequences were accepted.
- cDNA inserts did not contain sites for the restriction endonucleases BamH I, EcoR I, Hind III and Sph I, because these enzymes were used for *in vitro* RNA transcription. Exceptions to this were IL-1β and IL-4 that could not be designed without a Hind III and EcoR I restriction sites respectively.

A schematic for the cloning strategy used in the construction of template plasmid DNA is shown in Figure 4.1, details of the cloning techniques used can be found in Section 2C. Specific details of the cloning strategy used for the cytokine cDNA clones are presented in Table 4.1. Once the panel of ovine cDNA templates were generated, they were analysed by restriction digest with EcoR I, exceptions were the TNFα and IL-4 clones which were digested with EcoR I & Hind III and BamH I and Hind III, respectively (Section 2C.8); these sites are unique to the multiple cloning site (MCS). The results are shown in Figure 4.2. The orientation and nucleotide sequence of each probe template was confirmed by sequencing, and can be found in

Figure 4.1 Generation of cytokine template cDNA clones used in the RNase protection assay. Cloned ovine cytokine cDNAs were sub-cloned into pGEM®T-easy using PCR and 'TA' cloning. The orientation of the cDNA inserts were verified by automated sequencing, those clones with inserts in a 5' – 3' direction were selected. The cDNA inserts were then amplified by PCR using vector-specific primers flanked with restriction sites allowing directional cloning into pGEM®11-ZF(+) in a 5' – 3' orientation. The relevant features of the vectors are shown.

Cloning vectors containing ovine cytokine cDNA inserts.

Amplify fragments of insert by PCR. PCR products purified and inserted into pGEM®T-easy using TA cloning.



Amplify insert by PCR
Purify and digest with BamH I and Sph I
Ligate into pGEM®-11-Zf(+)

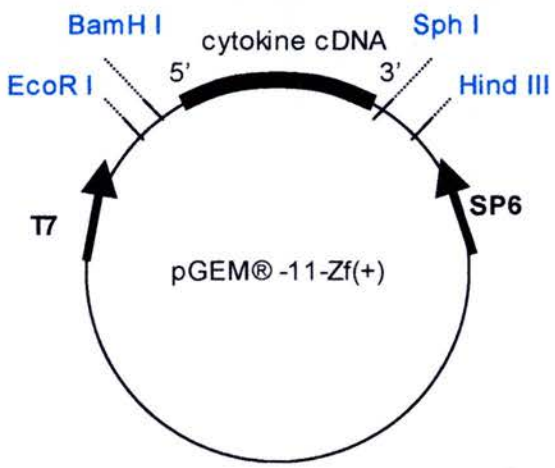


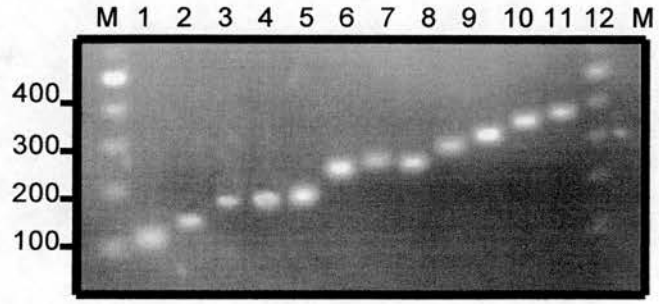
Table 4.1 Cloning strategy for development of riboprobe templates for the RPA

Clone	PCR primers / restriction endonuclease	Product length bp.
ovIL-1β (pBluescript II)	X54796F X54796R	123
ovIL-4 (pUC119)	M96845F M96845R	210
ovIL-6 (pTZ18)	X68723F X68723R	173
ovIL-8 (pBluescript II)	S74436F S74436R	348
ovIL-10 (pCRII)	Z29362F Z29362R	271
ovIL-12 (pCRII)	AF004024F1 AF004024R	332
ovIL-18 (pGEM-T-easy)	AJ401033F AJ401033R	160
ovGM-CSF (pBluescribe)	X53561F X53561R	296
ovIFNγ (pBluescribe)	X52640F X52640R	246
ovTGFβ (pGEM-T-easy)	X76916F X76916R	312
ovTNFα (pBluescript II)	Bam HI	148
ovGAPDH (pBluescript II)	AF030943F3 AF030943R3	98
ovATPase (pCR II)	X02813F1 X02813R2	115
pGEM-T-easy sub-clones	pGEM-XF pGEM-XR	vector MCS

PCR or endonuclease digest were used to generate templates for the RPA, as indicated. The Taq enzyme used in the PCR experiments was purchased from Promega (5 U / reaction). Templates for the PCR reactions were 10 ng of plasmid DNA. PCR primer sequences are listed in Appendix 4. PCR primers were designed to have similar annealing temperatures. Amplification parameters used in these reactions were: 94 0.5', 55 0.75', 72 0.5'; followed by 1 cycle of extension; 72 6'. Following analysis by agarose gel electrophoresis, PCR products were purified using a spin-bind column, or digested cDNA fragments excised and purified using a spin-bind column. DNA fragments were cloned into pGEM-T-easy using a 'TA' cloning system (Promega). Amplified plasmid DNA was purified using purification spin-bind columns (Qiagen), and positive clones sequenced to verify insert orientation. Clones in a 5' – 3' orientation were subjected to PCR using the vector specific primers pGEM-XF and pGEM-XR with incorporated restriction endonuclease sites Bam HI (pGEM-XF) and Hind III (pGEM-XR), and cloned into pGEM-11zf in a 5' - 3' orientation. The clones were verified by sequencing prior to use in the RPA. Sequences are presented in Appendix 6.

Figure 4.2 Analysis of sheep cytokine riboprobe template cDNA clones. Cytokine cDNA clones used for the transcription of cytokine riboprobes were analysed by restriction digest and gel electrophoresis. Following the digestion of 10 ng of plasmid DNA with Eco RI for the exception of the TNFa clone (EcoR I and Hind III) and IL-4 clone (BamH I and Hind III). Products were analysed by gel electrophoresis. Key for the lanes are shown in Table 4.2; insert size corresponds to the 'protected' length plus 30 nt. M = marker DNA.

Figure 4.2



Lane: Probe	Un-protected length (nt.)	Protected length (nt.)
1 GAPDH	159	98
2 IL-1β	179	123
3 TNFα	187	148
4 IL-18	227	160
5 IL-6	229	173
6 TGFβ	258	217
7 IL-4	266	210
8 IFNγ	300	246
9 IL-10	327	272
10 GM-CSF	351	288
11 IL-12 p40	386	332
12 IL-8	404	352

Table 4.2 The 'un-protected' and 'protected' sizes of the riboprobes. 'Protected' length refers to the nt. length of target mRNA bound by specific riboprobe, whereas 'un-protected' length refers to the nucleotide length of the riboprobe.

Appendix 6. The lengths of the 'un-protected' and 'protected' riboprobes are shown in Table 4.2.

4.2.1.2 The Generation of Antisense RNA Probes and Complementary RNA.

A schematic illustrating the relevant features of template DNA preparation and probe synthesis is presented in Figure 4.3. The RNA polymerase promoters T7 and SP6 were utilised for RNA synthesis. Following linearisation of the vector with the appropriate restriction endonuclease, the synthesis of antisense riboprobes was driven by SP6 polymerase; or sense RNA by T7 polymerase. When performing an RPA, radiolabelled antisense probes were hybridised with complementary sense synthetic RNA. This was utilised as a control for probe hybridisation and degradation. In general, a pool of sense RNA transcripts containing 10 pg of each RNA species was hybridised to the probe sets being used.

4.2.1.3 Sheep Cytokine Riboprobe Set.

As described in Figure 4.3, plasmid DNA templates were linearised with BamH I, and antisense probes synthesised using the SP6 promoter. A set of radiolabelled probes specific for sheep glyceraldehyde-3-phosphatase-dehydrogenase (GAPDH), adenosine triphosphatease (ATPase), IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, GM-CSF, IFNγ, TGFβ, and TNFα were produced and resolved on a sequencing gel. These are shown in Figure 4.4b (lanes 1 and 5). It was evident that a probe was missing from the set, and taking into account the spacing between the probes, the absent probe was either TNFα or IL-1β. The sequence of the TNFα template cDNA is shown in Figure 4.5. The BamH I site used for vector linearisation was situated adjacent to the 3' end of the cDNA insert. To establish whether the proximity of the cut site to the insert cDNA was a factor affecting the failure to generate a TNFα-riboprobe, an EcoR I site downstream of the BamH I site was used in an independent probe synthesis reaction. The products of two individual TNFα probe synthesis reactions, and an independent IL-1β probe synthesis reaction were run on a gel along with the probe set synthesised previously. The results are shown in Figure 4.4b. It was clear that the IL-1β riboprobe was present in the original probe set, however the TNFα-specific probe was missing when the template

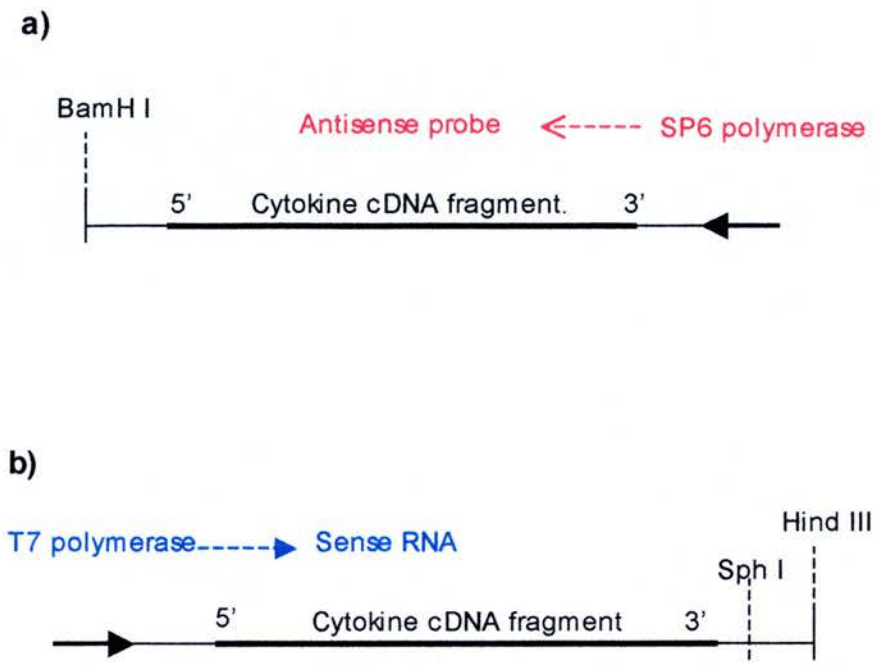
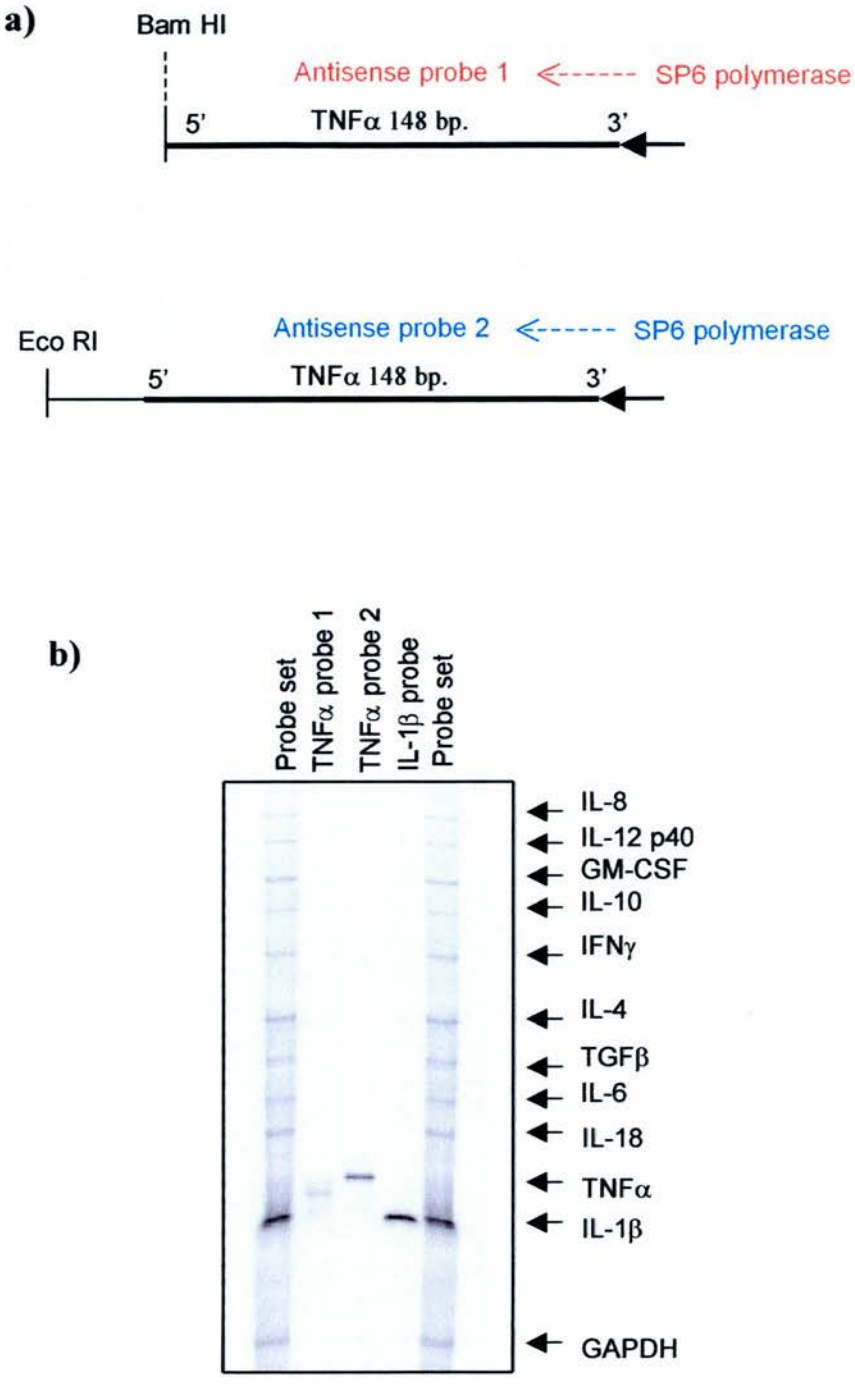


Figure 4.3 Synthesis of antisense riboprobes and complementary sense RNA.

a) Radiolabelled antisense probes are synthesised following linearisation of template cDNA clones with the restriction endonuclease BamH I. Transcription is driven by SP6 polymerase, and during synthesis, [^{32}P] UTP nucleotides are incorporated into the probe.

b) Complementary sense RNA is synthesised and used as target for the hybridisation of radiolabelled probes. This provides a positive control for hybridisation. cDNA were linearised with Hind III, except for the IL-1β clone which contains an internal Hind III site. In this case, the restriction endonuclease Sph I was used to linearise the vector DNA.

Figure 4.4 Un-protected probe set in the RNase protection assay. a) The cloning vector pGEM®11-Zf(+) containing a TNFα cDNA insert was linearised using BamH I or EcoR I restriction endonucleases, and used as template for the transcription of antisense radiolabelled RNA probes using the SP6 promoter **b)** A set of radiolabelled probes for ovine cytokines were synthesised from plasmid DNA templates linearised with the endonuclease BamH I. Individual probe synthesis reactions were performed for TNFα and IL-1β; templates were linearised using BamH I (TNFα probe 1; IL-1β probe) or EcoR I (TNFα probe 2). The sizes of the TNFα and IL-1β probes are 187 nt and 179 nt respectively.


a)
Bam HI
Antisense probe 1
SP6 polymerase
5'
TNFα 148 bp.
3'
Eco RI
Antisense probe 2
SP6 polymerase
5'
TNFα 148 bp.
3'
b)
Probe set
TNFα probe 1
TNFα probe 2
IL-1β probe
Probe set
IL-8
IL-12 p40
GM-CSF
IL-10
IFNγ
IL-4
TGFβ
IL-6
IL-18
TNFα
IL-1β
GAPDH

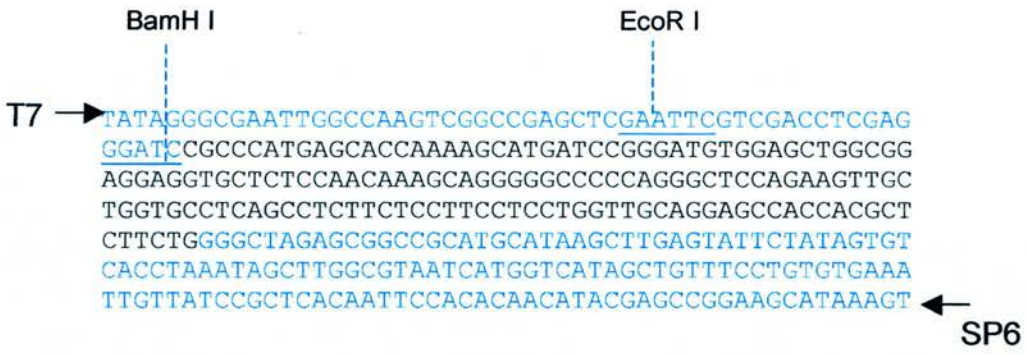


Figure 4.5 Nucleotide sequence of TNFα sub-clone. A fragment of ovine TNFα cDNA was cloned into the vector pGEM®11-Zf (+) in 5' – 3' orientation. The vector sequence is shown in blue, TNFα in black. Sense RNA is synthesized using the T7 promoter, antisense riboprobes from the SP6 promoter. To perform and *in vitro* RNA transcription, the vector DNA is linearised using appropriate restriction endonucleases. The restriction sites for BamH I and EcoRI are shown. Linearisation using BamH I failed to produce a riboprobe, whereas the linearisation using EcoR I successfully produced a TNFα specific probe (Figure 4.4).

DNA was linearised using BamH I. In subsequent experiments, the plasmid template used for the synthesis of the TNFα-specific probe was linearised with EcoR I, whereas the plasmid clones used as templates for the synthesis of other probes in the set (GAPDH, ATPase, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, GM-CSF, IFNγ, and TGFβ) were linearised with BamH I.

4.2.2 Standardisation of the RPA.

4.2.2.1 Transcript quantification.

An experiment was performed to investigate the quantitative nature of the RPA. Alveolar macrophages were cultured for 4 hours with 1 μg/ml *Salmonella typhimurium* LPS, the RNA extracted and various amounts of the RNA (2.5 μg, 5 μg, 7.5 μg, 9.5 μg) hybridised against a probe set containing GAPDH, IL-1β, IL-10, IL-8 and IL-18. Un-bound RNA (single-stranded) was digested with RNase T1 and RNase A, and 'protected' hybridised species resolved by PAGE. Following resolution on a sequencing gel and phosphorimaging, the intensity of the bands was evaluated using ImageMaster 3D software (Pharmacia), as described in Section 2C.12.8. The results are shown in Figure 4.6. Band intensity is expressed as arbitrary units of pixel density. As shown in Figure 4.7, the intensity, or volume of the GAPDH protected probes increases when increasing amounts of total RNA are used, and as such correlate with quantity of target RNA. This correlation was also evident for the IL-1β and IL-10 protected riboprobes (Fig. 4.7). However, the volume of the IL-8 protected probe did not vary between samples and appeared to have reached peak threshold levels on the gel exposure (Fig. 4.6). Band volumes of each protected probe were normalised against the GAPDH band in each sample, and expressed as a ratio of GAPDH levels (arbitrary units) in that sample, these are also shown in Figure 4.7b. Normalised values for IL-1β mRNA and IL-10 mRNA are similar for each sample, as would be expected from an individual RNA pool, whereas the normalised value of the IL-8 probes varied between samples reflecting small differences in band volume between the samples (Fig. 4.7b). Bands corresponding to IL-18 protected probes were not detected in any of the samples (Fig. 4.6). To determine if IL-18 transcripts were present in that RNA sample, an

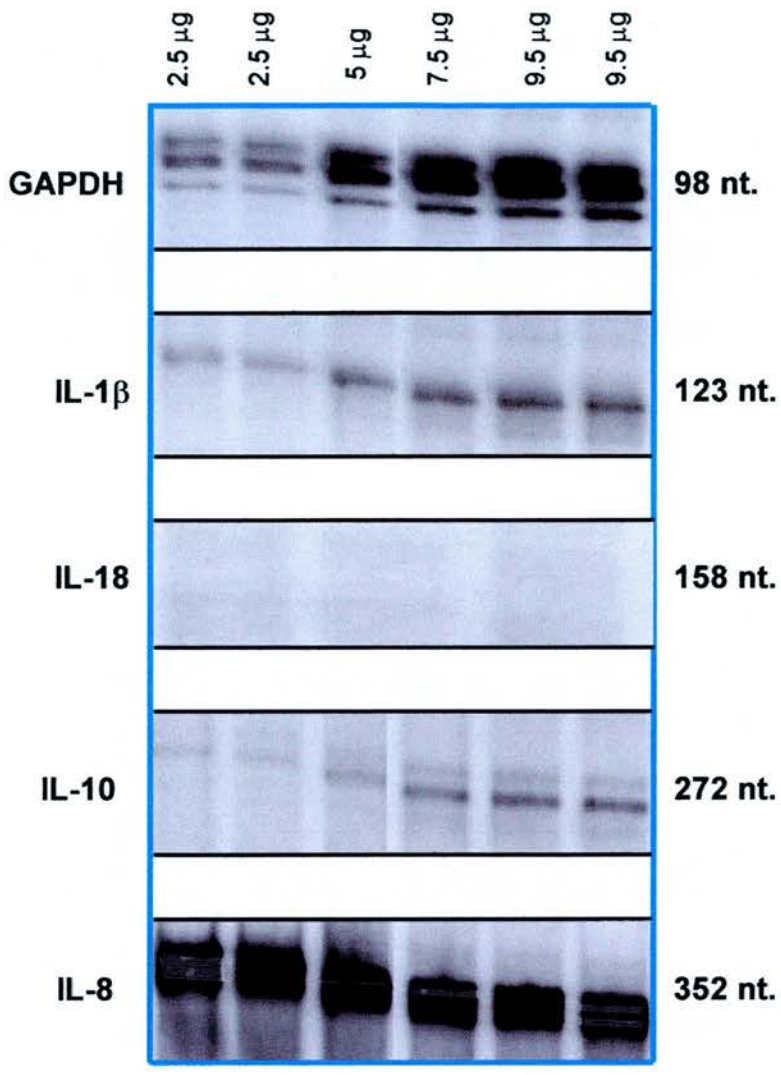
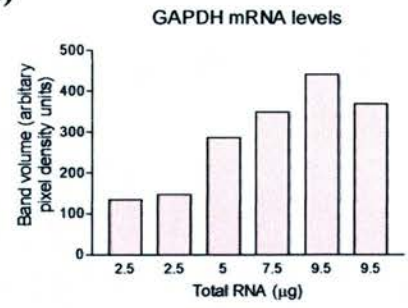
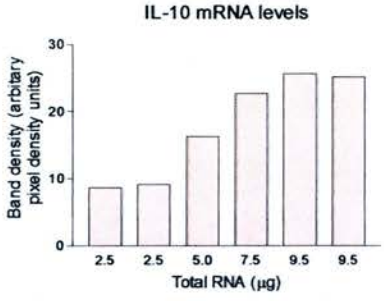


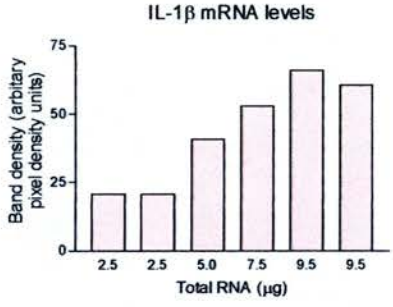
Figure 4.6 Quantitative evaluation of the RNase protection assay. Antisense radiolabelled probes for GAPDH, IL-1β, IL-18, IL-10 and IL-8 were hybridised with 2.5 µg – 9.5 µg total RNA as indicated with each lane, extracted from alveolar macrophages after 4 hours culture with 1 µg/ml LPS. After RNase treatment, the RNA-protected probes were PAGE-resolved and visualised by imaging on a phosphorimaging screen. The sizes of the protected probes span 352 nt. (IL-8) to 98 nt. (GAPDH).

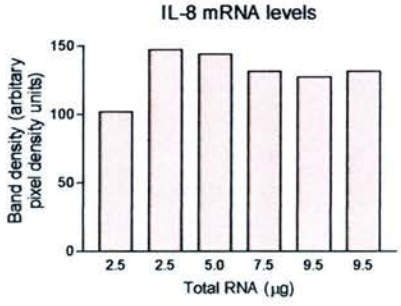
Figure 4.7 Determination of the quantitative nature of the RNase protection assay. The RPA was used to evaluate the intensity of transcripts for IL-10, IL-1β, IL-18, IL-8 and GAPDH in the same RNA sample using increasing amounts of target RNA. (a) Band volumes are expressed using arbitrary densitometric units. (b) The intensity of message is expressed as arbitrary densitometric units relative to GAPDH mRNA.

a)

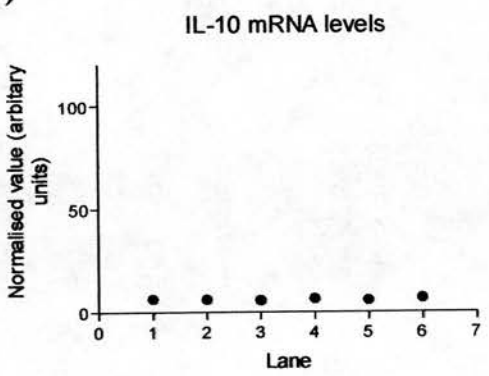

GAPDH mRNA levels
Band volume (arbitary pixel density units)
500
400
300
200
100
0
2.5 2.5 5 7.5 9.5 9.5
Total RNA (µg)

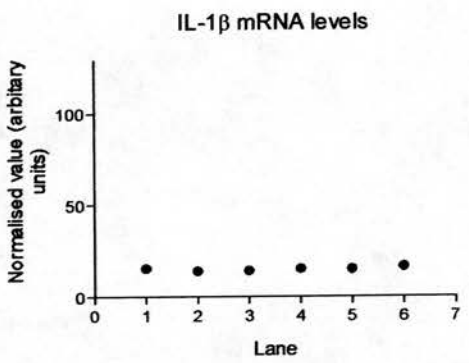

IL-10 mRNA levels
Band density (arbitary pixel density units)
30
20
10
0
2.5 2.5 5.0 7.5 9.5 9.5
Total RNA (µg)

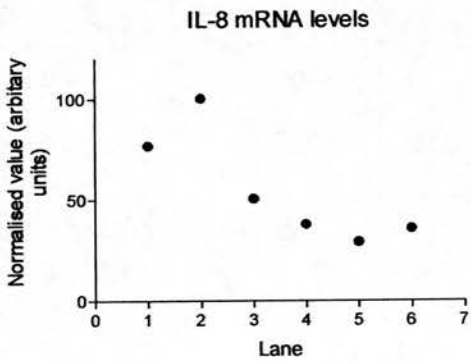

IL-1β mRNA levels
Band density (arbitary pixel density units)
75
50
25
0
2.5 2.5 5.0 7.5 9.5 9.5
Total RNA (µg)


IL-8 mRNA levels
Band density (arbitary pixel density units)
150
100
50
0
2.5 2.5 5.0 7.5 9.5 9.5
Total RNA (µg)

b)


IL-10 mRNA levels
Normalised value (arbitary units)
100
50
0
0 1 2 3 4 5 6 7
Lane


IL-1β mRNA levels
Normalised value (arbitary units)
100
50
0
0 1 2 3 4 5 6 7
Lane


IL-8 mRNA levels
Normalised value (arbitary units)
100
50
0
0 1 2 3 4 5 6 7
Lane

RT-PCR reaction was performed. This generated a product of expected size (Fig. 4.8), demonstrating that IL-18 mRNA was present in the RNA sample, but was below the level of detection using the RNase protection assay.

4.2.2.2 Detection of IL-18 mRNA by the RPA

In order to determine the threshold of detection of the hybridised IL-18 probe in the RPA, synthetic complementary RNA was synthesised and various amounts were hybridised with a probe set containing IL-18. The results are shown in Figure 4.9. A single band of approximately 158 nt. was detected, demonstrating the specificity of the IL-18 riboprobe. Bands were clearly evident when 50 pg – 1 pg of target complementary RNA was used. Although a faint band was evident on the gel exposure image presented (Fig. 4.9) using 1 pg of positive control RNA, this band was easily detected by the software package used for analysis of the RPA gels. Therefore, this experiment demonstrated that the RNase protection assay has a sensitivity threshold of detecting 1 pg of IL-18 RNA.

4.2.3 Analysis of Cytokine Expression in Alveolar Macrophages (AM)

4.2.3.1 AM Culture and Stimulation

An initial set of experiments were performed to establish a protocol for *in vitro* LPS stimulation and to determine the number of adherent bronchoalveolar cells required to obtain sufficient amounts of RNA for RPA analysis. Bronchoalveolar (BAL) cell culture is described in Section 2A.1, cells were cultured with or without LPS (1 μg/ml) and adherent cells were recovered following culture durations of 1, 4, 6, 16 or 24 hours. Cell yield and viability were determined over the time course, the results are shown in Figure 4.10a and 4.10b respectively. There was a gradual decrease in the number of adherent cells recovered over 24 hours. The percentage of viable cells fluctuated over time, reaching a minimum of 53% in LPS stimulated cells after 16 hours culture. Consequently, RNA yields also differed, and are shown in Figure 4.10c.

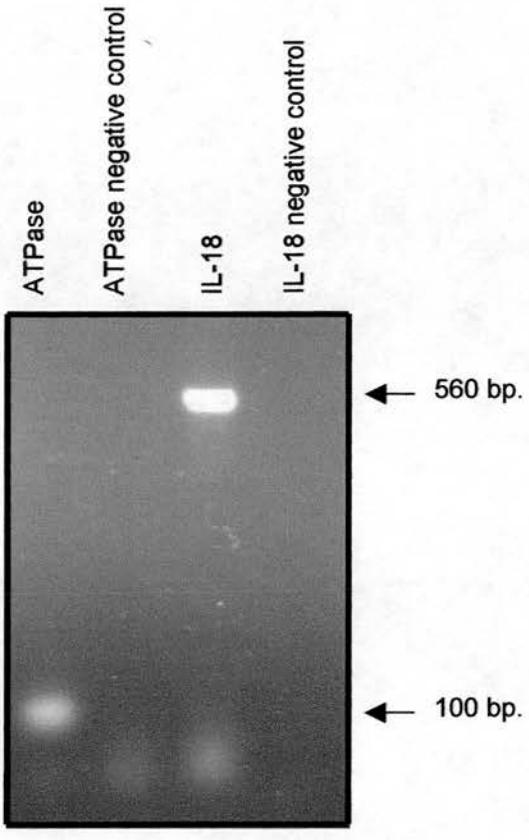


Figure 4.8 Analysis of IL-18 expression by alveolar macrophages stimulated with LPS. RNA extracted from alveolar macrophages after 4 hours culture with 1 μg/ml LPS was subject to RPA analysis (Fig. 4.6) and RT-PCR amplification. RNA was reverse transcribed in the presence of an oligo (dT) primer, then 10% of the cDNA sample was subject to PCR using primers specific for ATPase or IL-18. The primer sequences are listed in Appendix 3. PCR reactions were performed omitting template DNA to control for reagent contamination (negative controls). Analysis of the PCR reactions are shown. The IL-18 primers amplify a region of cDNA 560 bp., the amplicon is indicated with an arrow. The ATPase amplicon was 100 bp. in length and is also indicated.

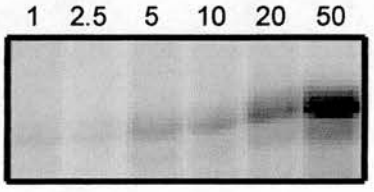


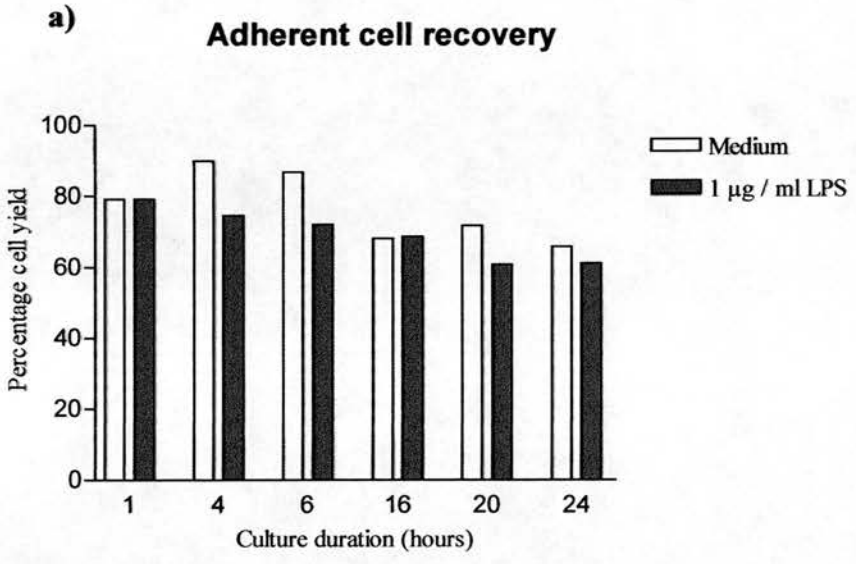
Figure 4.9 Sensitivity of the RNase protection assay for the detection of IL-18 transcripts. A probe set containing radiolabelled probes specific for IL-18, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, GM-CSF, IFNγ, TNFα, TGFβ, GAPDH was hybridised with 1 – 50 pg. of synthetic sense IL-18 RNA. The amount (pg.) of target RNA in the sample is shown with each lane. Samples were RNase treated and resolved on a sequencing gel. The size of the protected band shown was 158 nt.

Figure 4.10 Bronchoalveolar cell culture. Cells were recovered by bronchoalveolar lavage and cultured at 10^6 cells / ml with culture medium or 1 μg/ml bacterial lipopolysaccharide (LPS). 4.5 x 10^6 cells were cultured for each time point. Following culture the adherent cells were washed twice with PBS and recovered using 5mM EDTA.

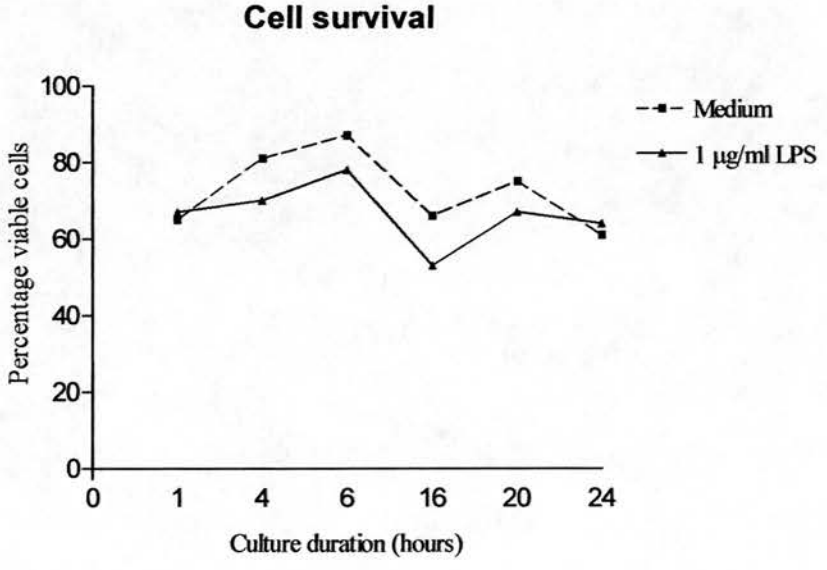
(a) Cell number and viability were determined by trypan blue exclusion, and analysed microscopically, these are shown in graphs (a) and (b).

(c) RNA was extracted from the cells using RNAzol B, the quality and quantity determined as described in Section 2C.1.2. The purity, as determined using spectrophotometry had a A_{280}/A_{260} ratio of over 1.9 The 18S and 28S ribosomal bands were evident by agarose gel electrophoresis, indicating the intact nature of the RNA. Amounts of total RNA recovered are shown.

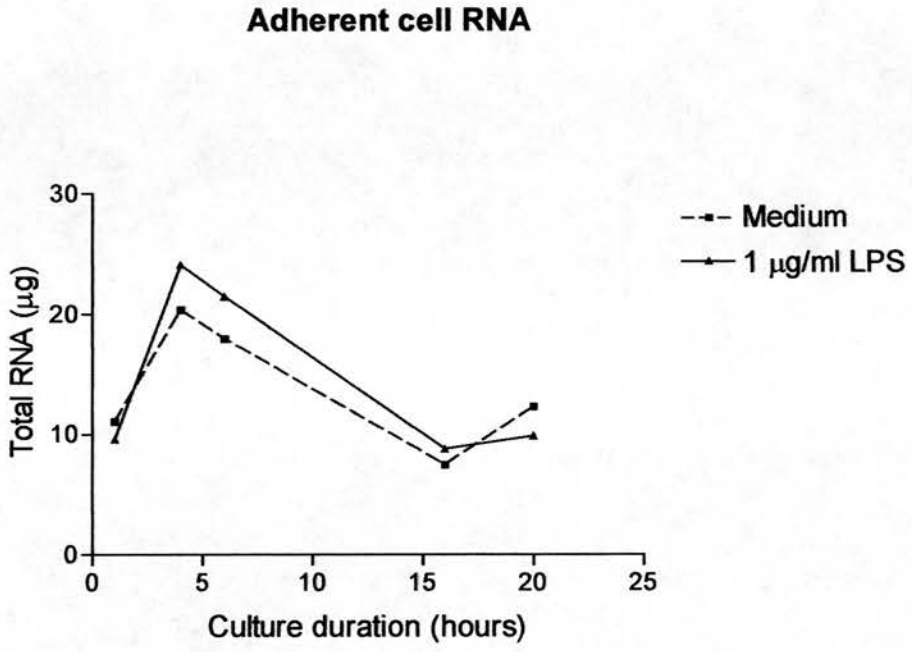
a)


Adherent cell recovery
Percentage cell yield
100
80
60
40
20
0
1
4
6
16
20
24
Culture duration (hours)
Medium
1 µg / ml LPS

b)


Cell survival
Percentage viable cells
100
80
60
40
20
0
0
1
4
6
16
20
24
Culture duration (hours)
Medium
1 µg/ml LPS

c)


Adherent cell RNA
Total RNA (µg)
30
20
10
0
0
5
10
15
20
25
Culture duration (hours)
Medium
1 µg/ml LPS

4.2.3.2 Time-course Experiments

Transcripts expressed at low levels may be below the level of detection by the RPA, as demonstrated for IL-18 (Section 4.2.2.2.). It was therefore important to recover similar amounts of RNA from the cells at each time point so that the sensitivity of the assay would be comparable between samples. Considering the decrease in amount of RNA isolated from the cells over 20 hours (Fig. 4.10c), twice the number of cells were used for the 16 hour and 20 hour cultures in subsequent experiments. Each experiment was performed on BAL cells recovered from an individual animal. Cells were cultured in medium supplemented with 1 μg/ml LPS; cells cultured in medium alone were included to control for the affects of culture. Experimental groups and culture durations are illustrated in Table 4.3.

Table 4.3 Experimental protocol for time-course experiments on bronchoalveolar cells

Experimental group	Volume cells (x)[a]	Culture time
A	1(x)	1 hour
B	1(x)	4 hours
C	1(x)	6 hours
D	2(x)	16 hours
E	2(x)	20 hours

[a] volume of cell suspension of $1x10^6$ cells / ml divided by 14 – stimulated and control cells; five experimental groups.

4.2.3.3 Presentation of Data

The phosphorimages of the denaturing gels were transported into the ImageMaster 3D package (Pharmacia). Standard curves were produced from the un-hybridised probes by plotting probe size (nt.) (Log_{10} scale) against distance migrated in the gel (linear scale); examples are shown later in Figs. 4.13 and 4.25. The migration distance of the probe : sample RNA hybrids ('protected' bands) were determined from the standard curve. Band densities were then evaluated (Section 2C.12.8). Mean values of band density expressed as a ratio of GAPDH mRNA

levels (normalised values) were plotted. In many cases the number of replicate samples were low (n = 1 – 4), so standard errors were not presented. Graphical presentation of the effects of LPS stimulation on the expression of each transcript at each time point was achieved by plotting the normalised value of each cytokine mRNA in LPS stimulated versus control cells on a $\log_{10}$ scale, and are shown in Appendix 7. This illustrates fold-differences between the experimental groups. Where no bands were detected in the control or stimulated cells, a value of 0 was given, and were consequently not included in this type of analysis. The data from the sheep in each experiment were pooled, and student T tests were performed. It should be noted that with such a small sample group (n=4), the p values were not stringent, but gave an indication of the effects of LPS stimulation on the cells.

4.2.4 Cytokine Expression in AM Prior to Culture

Macrophages enriched from bronchoalveolar lavage (BAL) cells (Section 2A.3) derived from sheep 1 were analysed by RPA. To inhibit RNA transcription during the separation procedure, actinomycin D was added to the buffers. Duplicate samples of 10 μg of RNA were subject to the RPA, the results from this one experiment are shown in Figure 4.11. Transcripts for IL-8, IL-1β, IL-18 and TGFβ were detected. The expression of IL-8 was the highest of the cytokines detected, whereas IL-1β mRNA levels were the lowest.

4.2.5 Cytokine mRNA Expression by Adherent Bronchoalveolar (BAL) cells

In the first series of time course experiments, the kinetics of cytokine mRNA expression by BAL cells was analysed by RPA following exposure to 1 μg/ml LPS for 1 hour, 4 hours, 6 hours, 16 hours and 20 hours. RNA was recovered from the adherent cells. Cells derived from three donor animals were used, duplicate samples of approximately 15 μg of RNA were analysed from sheep 1, whereas single samples of around 20 μg of RNA were analysed from sheep 2 and sheep 3. Figure 4.12 shows the results of the RPA analysis of samples from sheep 1. Expression kinetics of individual cytokine mRNAs over the time course of LPS treatment are shown in Figures 4.13 – 4.24. Due to a sample handling error, no data is available from sheep 2 and 3 at the 20 hours time point of LPS stimulation.

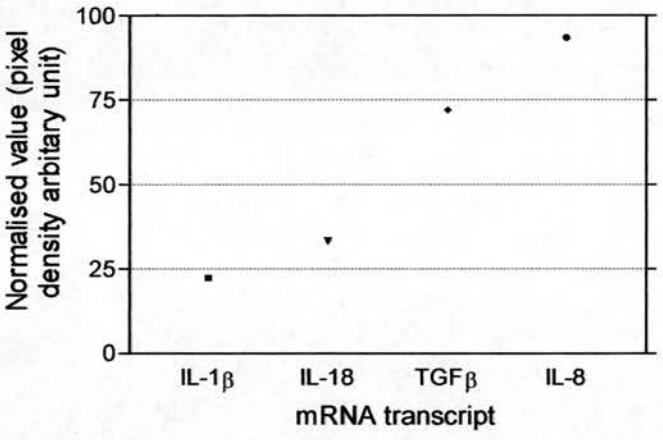
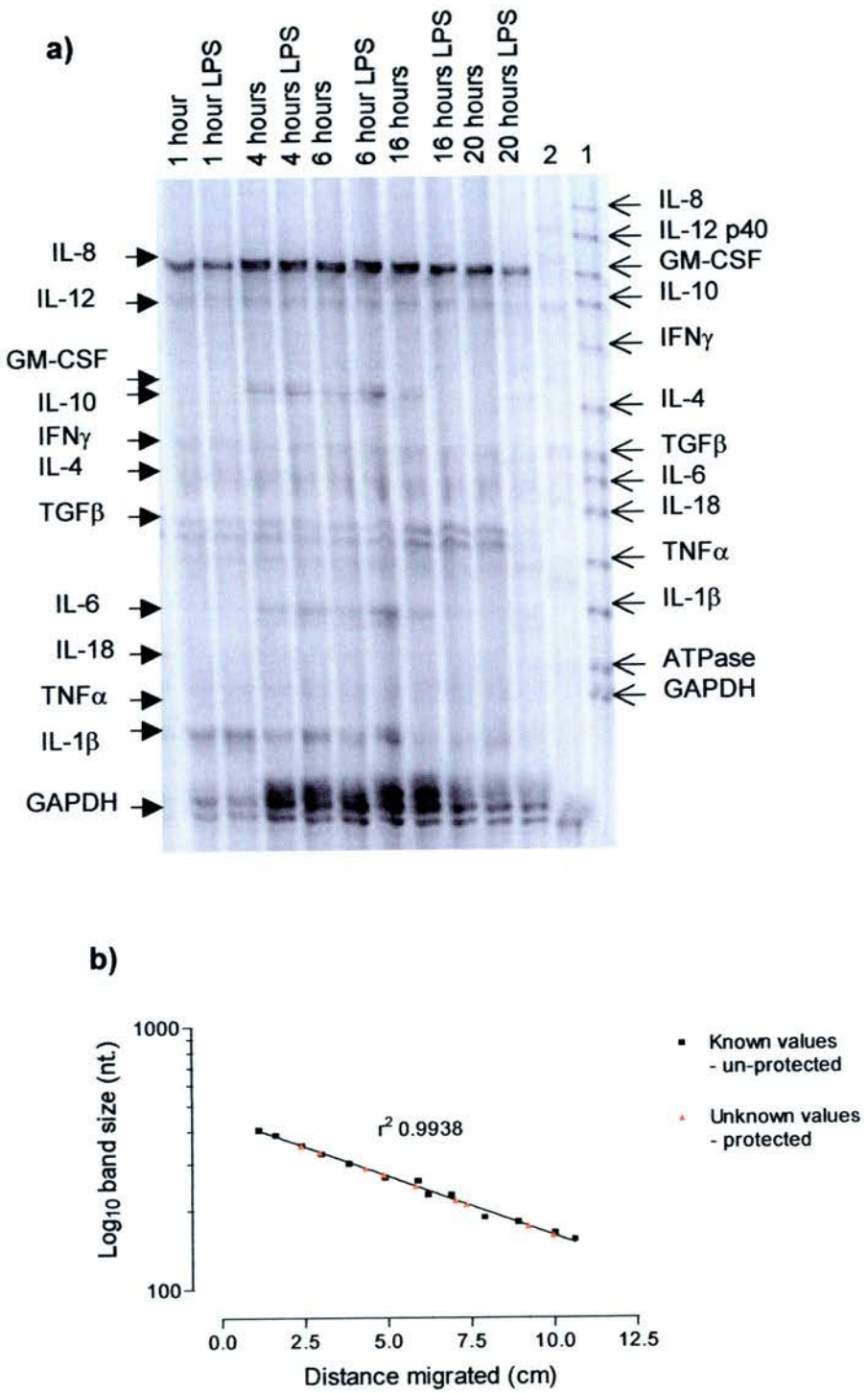


Figure 4.11 Expression of cytokines in freshly isolated alveolar macrophages. Macrophages were enriched from sheep bronchoalveolar lavage cells in accordance to density (Section 2A.3). RNA was extracted and subjected to RPA analysis. The levels of expression of each transcript were determined by normalising against the levels of the housekeeping gene GAPDH. The results presented are the average of duplicate samples, in an experiment involving one animal.

Figure 4.12 Measurement of cytokine mRNA levels using the RNase protection assay. Bronchoalveolar cells were cultured over 20 hours with 1 μg/ml LPS, and the levels of mRNA's in the adherent cells evaluated using a RNase protection assay. The sizes of the unprotected probe set, shown in lane 1, spanned 404 nt (IL-8) to 154 nt (GAPDH). Lane 2: a synthetic set of sense complementary RNA was hybridised with the radiolabelled antisense probe RNA probes for the designated transcripts. At a range of time periods over 20 hours, RNA was extracted from the cultured adherent alveolar cells derived from sheep 1, and aliquots of approximately 15 μg were analysed with the RNase protection assay. The sizes of the protected probes span 352 nt (IL-8) to 98 nt (GAPDH).

b) The standard curve used to identify protected bands. Migration distance of un-protected probes was measured, and plotted against the nucleotide length using non-linear regression analysis. Curve goodness of fit is presented ($r^2 = 0.9938$). The position of the bands of interest (protected probes) on the gel were ascertained from the standard curve.


a)
1 hour
1 hour LPS
4 hours
4 hours LPS
6 hours
6 hour LPS
16 hours
16 hours LPS
20 hours
20 hours LPS
2
1
IL-8
IL-12
GM-CSF
IL-10
IFNγ
IL-4
TGFβ
IL-6
IL-18
TNFα
IL-1β
GAPDH
IL-8
IL-12 p40
GM-CSF
IL-10
IFNγ
IL-4
TGFβ
IL-6
IL-18
TNFα
IL-1β
ATPase
GAPDH
b)
1000
100
Log10 band size (nt.)
r2 0.9938
0.0
2.5
5.0
7.5
10.0
12.5
Distance migrated (cm)
Known values
- un-protected
Unknown values
- protected

Figures 4.13 – 4.24 Kinetics of expression of cytokine mRNA. The expression of a panel of cytokine mRNAs by adherent bronchoalveolar cells cultured with LPS was assessed using an RNase protection assay (see text for details). The mean normalised value of protected radiolabelled probes for individual cytokine transcripts were plotted in x-y graphs over a time course. A comparison of mRNA levels between medium controls and LPS stimulated cells is shown; the normalised value of the mRNA transcript in each sample (LPS and medium control) were plotted on a Log_{10} scale (y = LPS value, x = control value). This type of analysis shows the levels of mRNA in each sample analysed. An example is presented for IL-18 expression, the comparisons of expression of the other cytokine mRNA transcripts are in Appendix 7.

4.13 IL-18: kinetics of expression (1 – 20 hours). IL-18 transcripts were not detected in cells derived from sheep 1.

4.14 IL-18: comparison of control cells and LPS stimulated cells expression levels

4.15 IL-12 p40: kinetics of expression (1 – 20 hours)

4.16 IL-10: kinetics of expression (1 – 20 hours)

4.17 IL-6: kinetics of expression (1 – 20 hours)

4.18 GM-CSF: kinetics of expression (1 – 20 hours)

4.19 IL-8: kinetics of expression (1 – 20 hours)

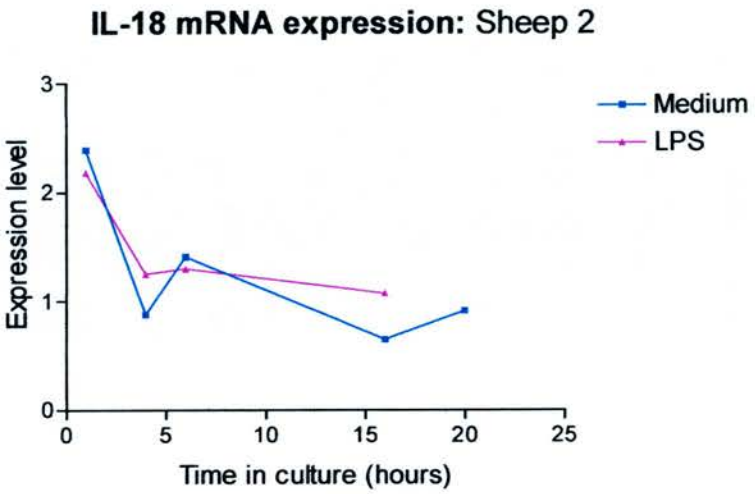
4.20 IL-1β: kinetics of expression (1 – 20 hours)

4.21 TNFα: kinetics of expression (1 – 20 hours)

4.22 TGFβ: kinetics of expression (1 – 20 hours)

4.23 IL-4: kinetics of expression (1 – 20 hours)

4.24 IFNγ: kinetics of expression (1 – 20 hours)



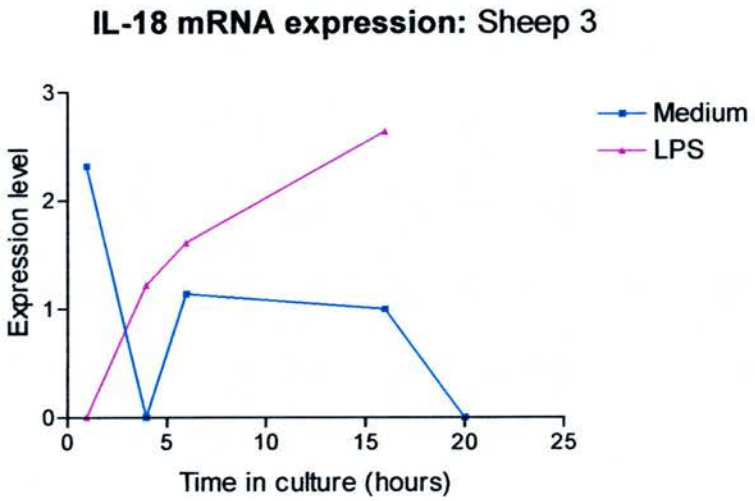


Figure 4.13 Expression of IL-18.

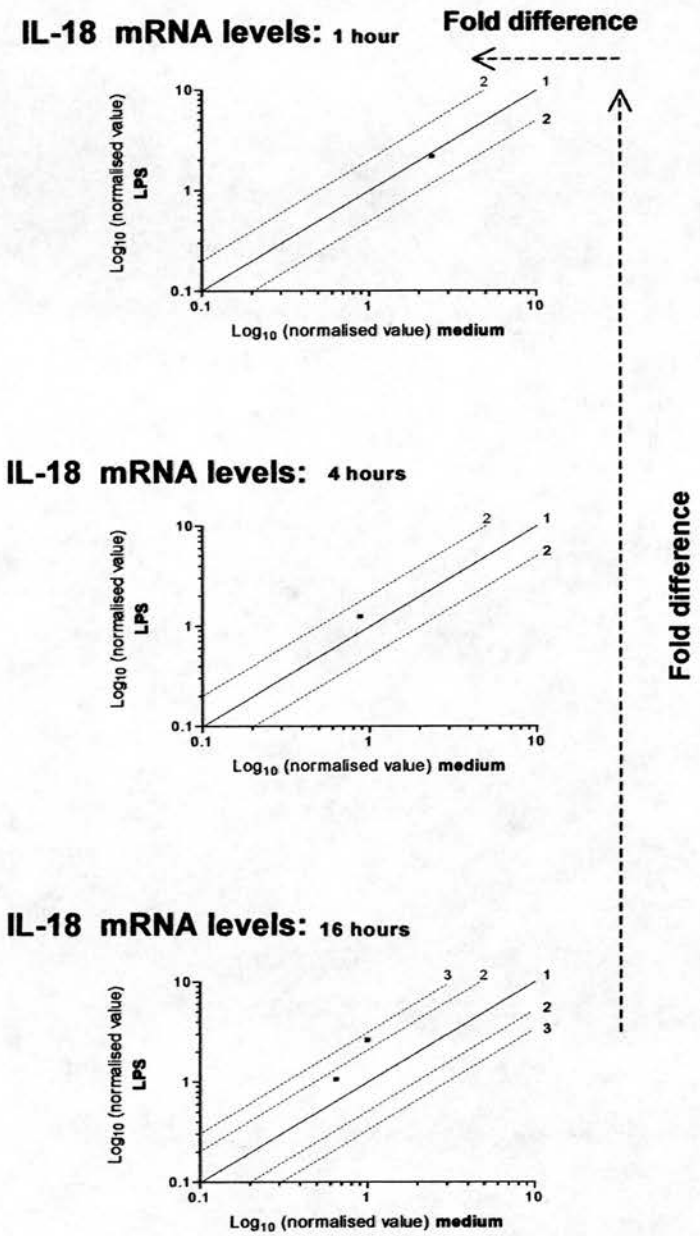
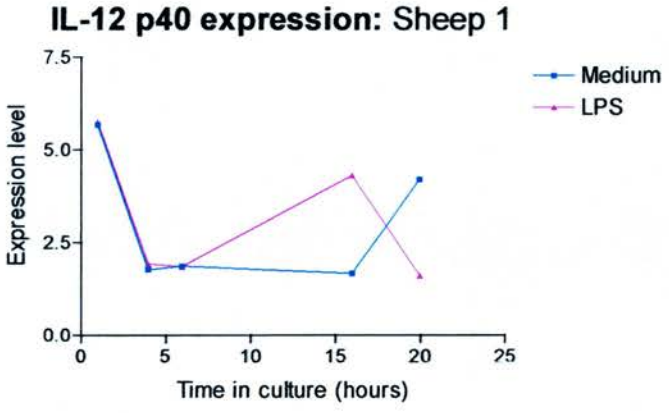
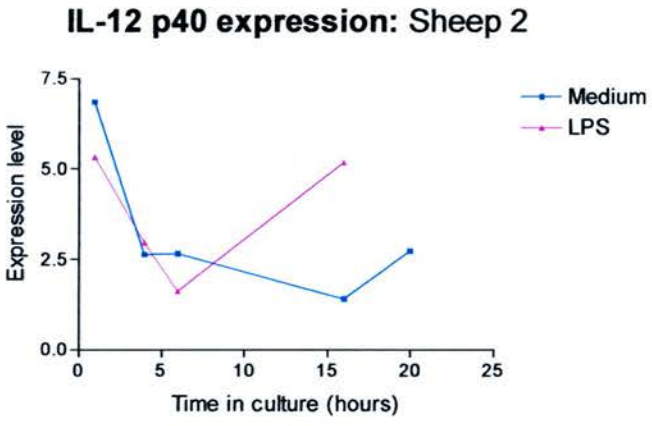


Figure 4.14 Expression of IL-18.





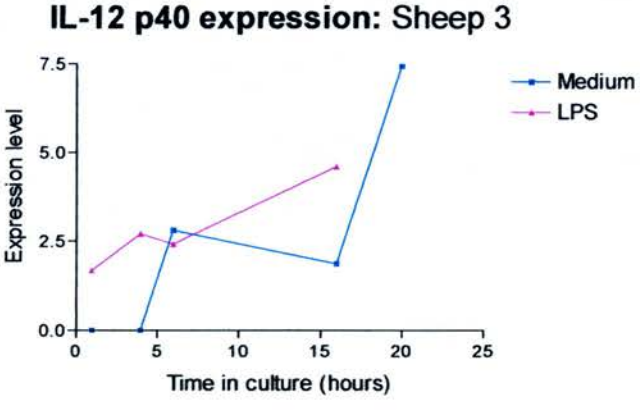
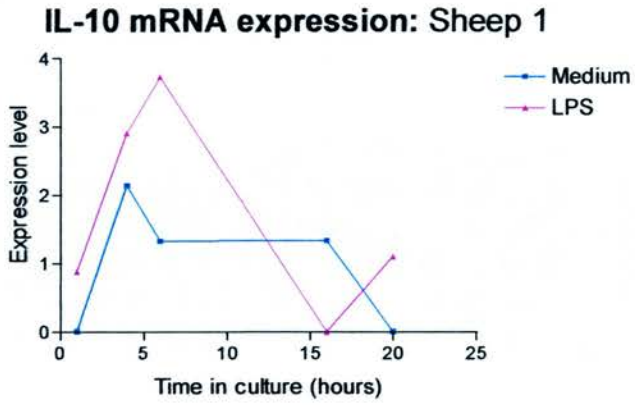
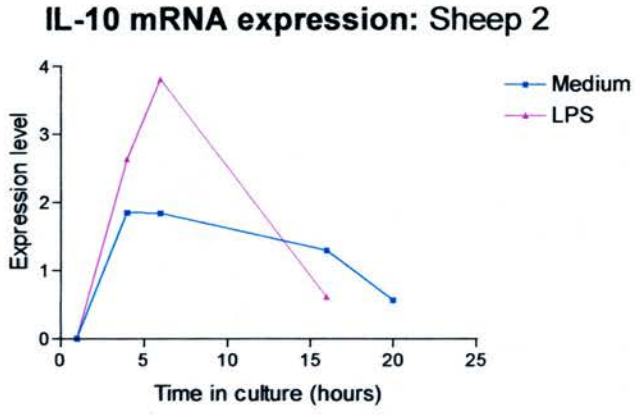


Figure 4.15 Expression of IL-12 p40.





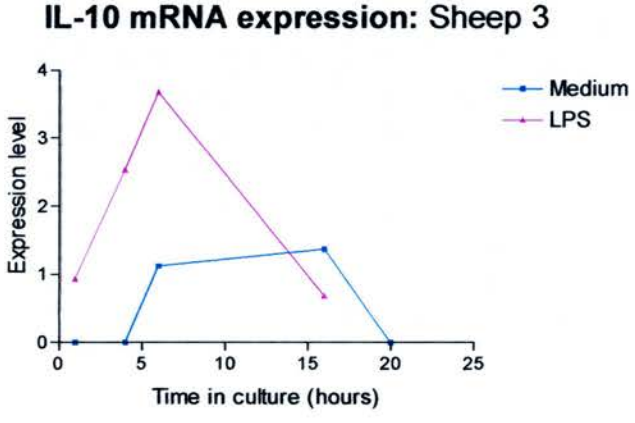


Figure 4.16 Expression of IL-10.

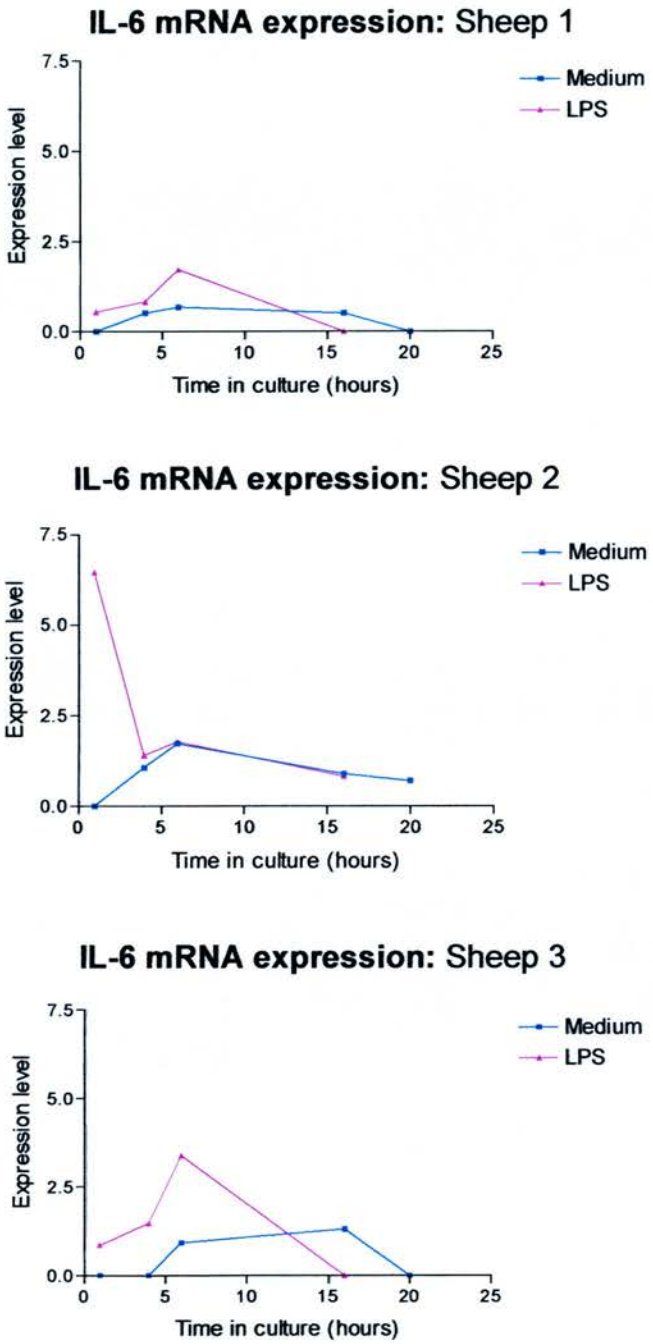
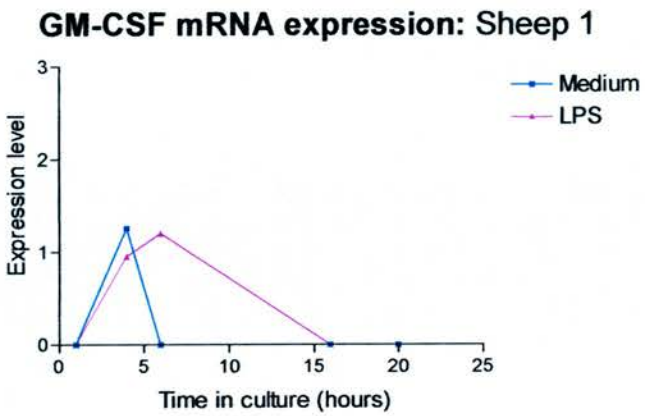
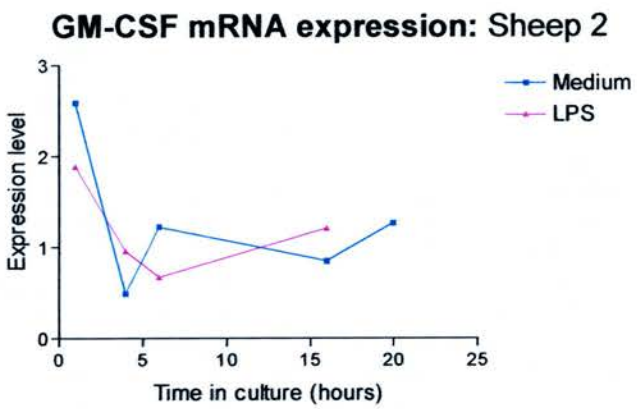


Figure 4.17 Expression of IL-6.





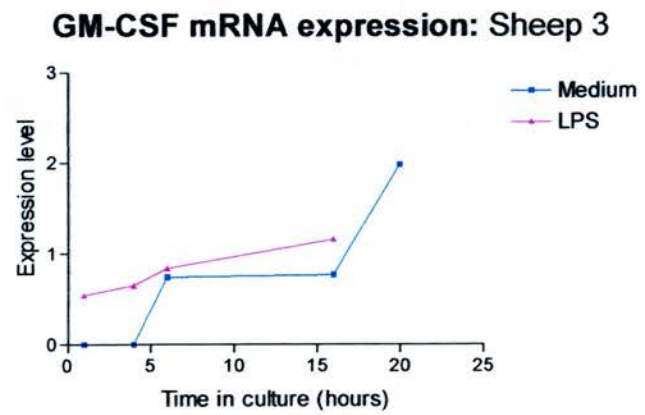


Figure 4.18 Expression of GM-CSF.

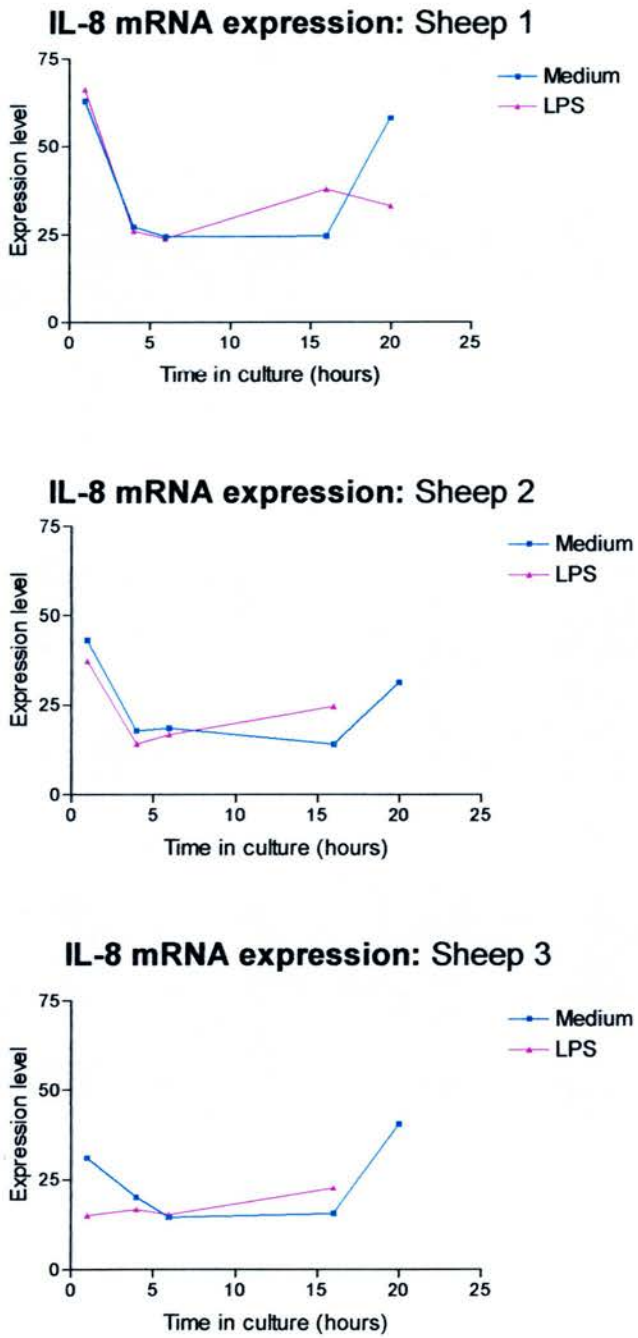
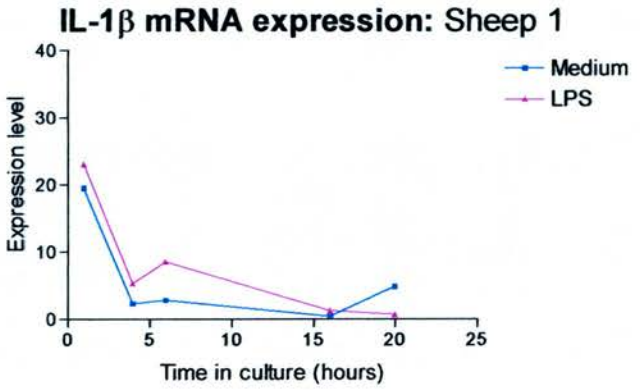
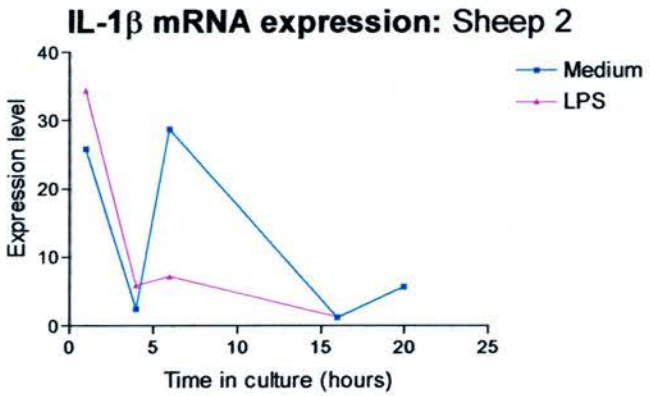


Figure 4.19 Expression of IL-8.





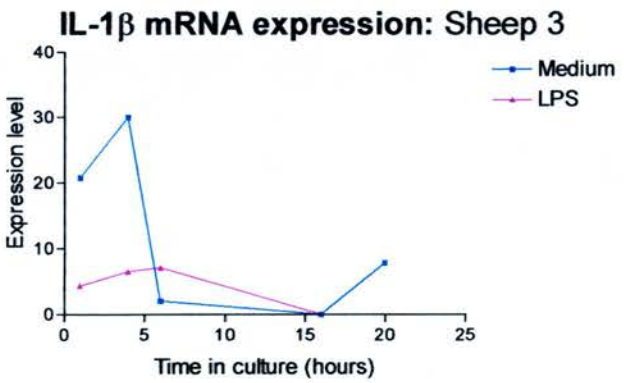
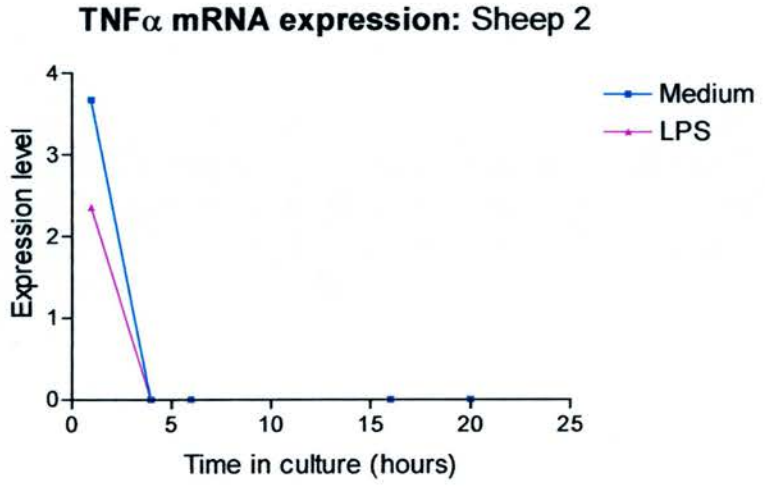


Figure 4.20 Expression of IL-1β.



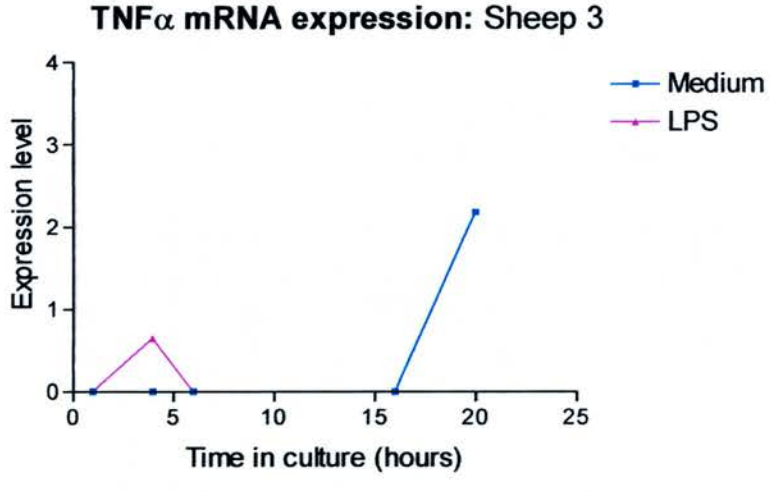
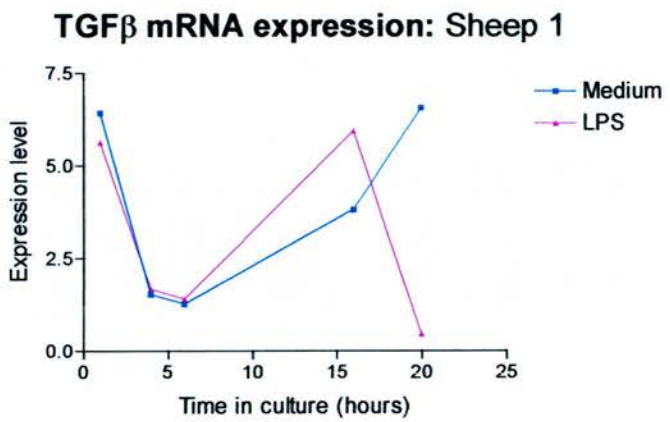
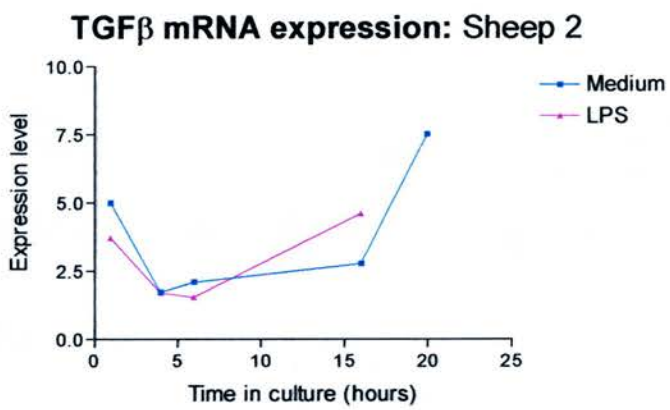


Figure 4.21 Expression of TNFα.





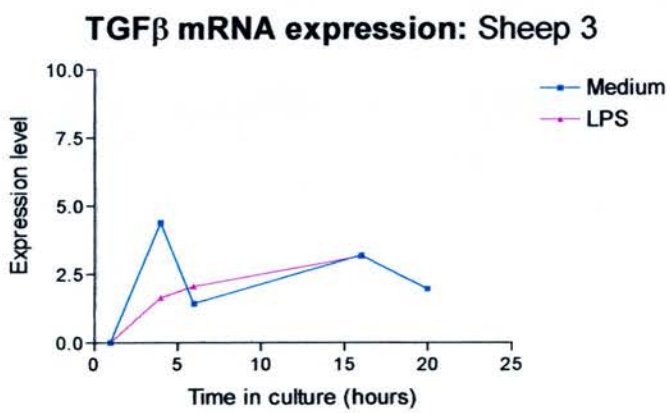
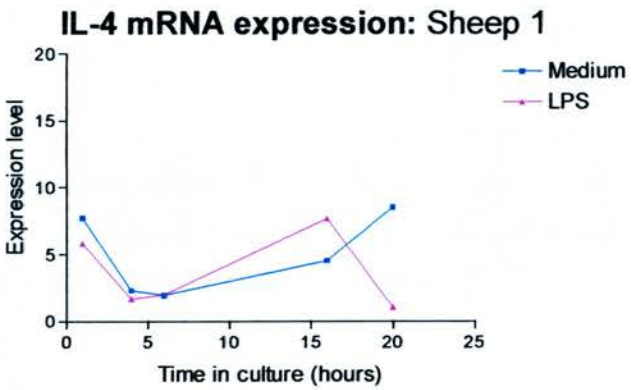
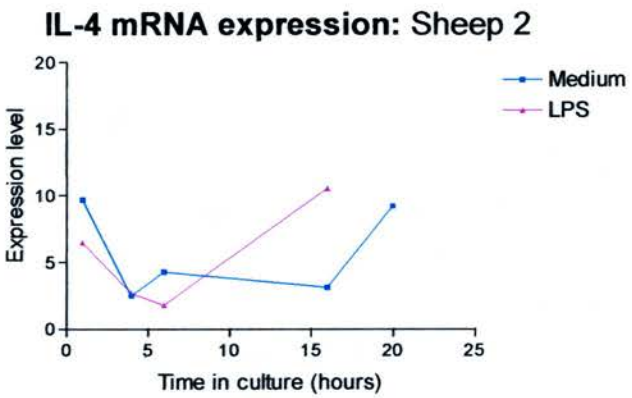


Figure 4.22 Expression of TGFβ.





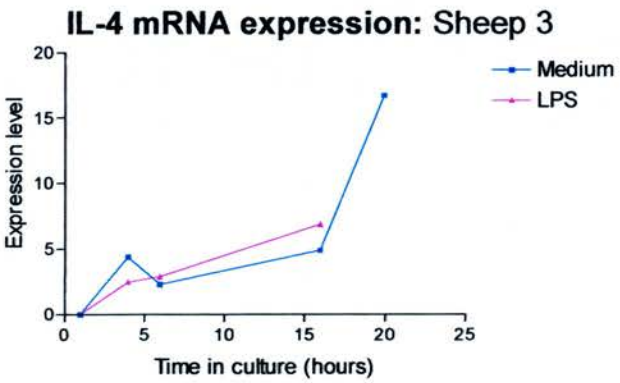


Figure 4.23 Expression of IL-4.

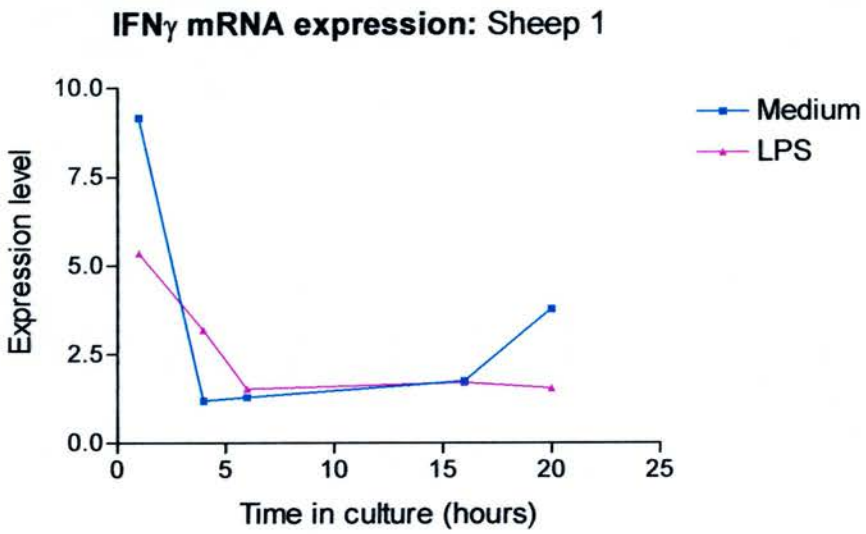


Figure 4.24 Expression of IFNγ.

4.2.6 Kinetics of Expression of Cytokine mRNA: Adherent BAL cells

There were no differences between cells derived from different sheep for the kinetics of expression of IL-18, IL-12, IL-6, IL-10, IL-8 and IL-4 over the 20 hour time-course (Figs 4.13, 4.15, 4.16, 4.17, 4.19, 4.23). Conversely, GM-CSF, IL-1β, TNFα and TGFβ were expressed with different kinetics between the animals (Figs. 4.19, 4.21, 4.22, 4.23). Expression of GM-CSF differed in each animal with high levels in both control and LPS stimulated BAL cells detected in sheep 2 after 1 hour culture, whereas none were detected in controls cells of sheep 1 and 3 and LPS stimulated cells of sheep 1 (Fig. 4.18). GM-CSF levels showed an upward trend in LPS stimulated cells from sheep 2 and 3, whereas they dropped to undetectable levels in sheep 1 over the same time period. Patterns of expression of IL-1β over 20 hours (Fig. 4.19) were similar in BAL cells from sheep 1 and 3. Control BAL cells from sheep 2 cultured in media alone expressed a spike of increased IL-1β levels at 6 hours culture, and this band was evident in all the samples (not shown). On the other hand, TGFβ expression kinetics differed in sheep 3 compared with sheep 1 and 2 (Fig. 4.22). Levels of TGFβ showed an upward trend between 6 hours and 20 hours culture in BAL cells derived from sheep 1 and sheep 2, whereas levels dropped in cells from sheep 3. Peak levels of TNFα mRNA were detected after 1 hour culture in cells from sheep 2, whereas maximal TNFα mRNA was detected after 20 hours culture in BAL cells from sheep 3 (Fig. 4.21). Levels of TNFα mRNA detected in both samples were very low; therefore differences may be enhanced at the low range of pixel density analysis. IFNγ was detected in cells from sheep 1 throughout the 20 hour time-course (Fig. 4.24).

Peak levels of most cytokine transcripts were detected after 1 hour in culture. Exceptions were IL-4, reaching maximal levels after 20 hours in control cells, and TGFβ and TNFα which reflected variations between cells from different sheep. Peak levels of TGFβ and TNFα were detected after 20 hours in at least one of the animals. IL-6 levels were the highest after 1 hour and 6 hours in LPS stimulated cells, and differences in the kinetics of GM-CSF were reflected in peak levels detected after 1, 4 and 20 hours in sheep 2, 1 and 3 respectively.

In general, LPS had minimal effects on the augmentation of cytokine expression compared with controls (Appendix 7a). LPS induced an increase in IL-18

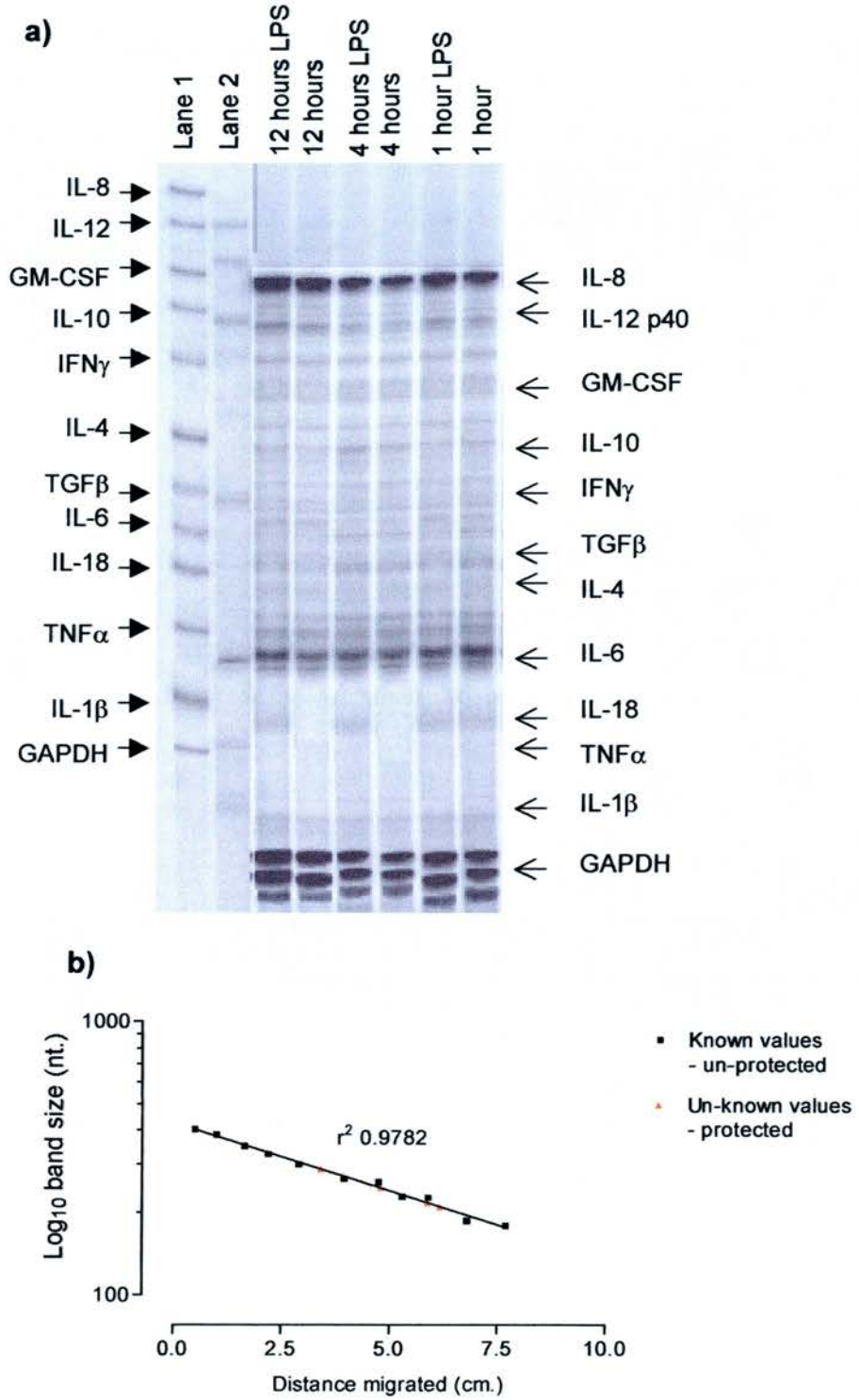
expression at the 16 hour time point only, averaging 2 fold higher than controls. Expression of IL-1β was generally higher in LPS stimulated cells over 1 – 16 hours compared with controls. However, LPS failed to induce significant enhancements of IL-4, IL-6, IL-8, IL-10, GM-CSF, IFNγ, TGFβ and TNFα expression.

It was evident that the adherent BAL cells expressed similar levels of cytokine mRNA in response to culture with medium alone and with 1 μg/ml LPS. Classically, LPS induces enhanced expression of pro-inflammatory cytokines, particularly IL-1β and TNFα, and others such as IL-6 and IL-12 p40, so the negligible effects of LPS in these experiments was surprising. This may have been a consequence of culture of the mixed population of BAL cells. Macrophages comprise 83% of the cells obtained by bronchoalveolar lavage of sheep; lymphocytes make up 5%, and polymorphonuclear cells (PMNs) and mast cells the remainder (Lujan et al., 1993). It was possible that the presence of other cell types, such as lymphocytes, had effects on the responses of the adherent cell population. In addition, transcripts for IL-4 and IFNγ were detected in LPS stimulated and control cells, indicating the presence of activated lymphocytes (T cells producing IFNγ and IL-4) and other populations such as NK cells (producing IFNγ) or mast cells (producing IL-4) in the cultures.

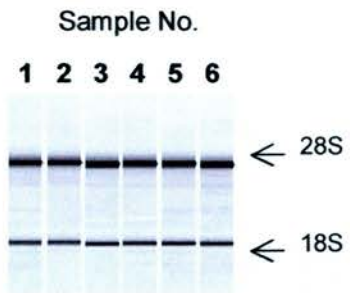
4.2.7 Measurement of Cytokine Expression by AM

In the second series of experiments, alveolar macrophages were purified by adherence in culture overnight before beginning the time course. Non-adherent cells were removed by washing and the remaining adherent cells then cultured with or without 1 μg/ml LPS for 1 hour, 4 hours and 12 hours. Evaluation of cytokine expression by RPA, and the analysis and presentation of data were repeated as in the previous set of experiments. The data presented below represents two experiments involving two animals. Three or four samples of 15 – 20 μg of RNA from sheep 4 were analysed, whereas duplicate samples of 15 μg of RNA were analysed from sheep 5 for each time point. Figure 4.25 shows the phosphorimage of samples from sheep 4 after being subject to the RNase protection assay. The banding pattern on

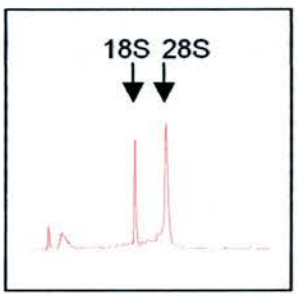
Figure 4.25 Measurement of cytokine mRNA levels in alveolar macrophages using the RNase protection assay. Alveolar macrophages were purified by adherence and then cultured over 12 hours with 1 μg/ml LPS. Cytokine mRNA levels were evaluated using a RNase protection assay (RPA). The unprotected probe set is shown in lane 1, sizes of the probes span 404 nt (IL-8) to 154 nt (GAPDH). Lane 2: synthetic complementary RNA was hybridised with the radiolabelled antisense probe RNA probes for the designated transcripts. At a range of time periods over 12 hours, RNA was extracted from the alveolar macrophages derived from sheep 4, and aliquots of 15 – 20 μg were analysed with the RPA. The sizes of the protected probes span 352 nt (IL-8) to 98 nt (GAPDH).
b) The standard curve used to identify protected bands. The migration distance of un-protected probes is measured, and plotted against the nucleotide length using non-linear regression analysis. Curve goodness of fit is presented ($r^2 = 0.9782$). The position of the bands of interest (protected probes) on the gel are ascertained from the standard curve.
c) The macrophage RNA samples were analysed by agarose gel electrophoresis prior to commitment to RPA analysis; 18S and 28S ribosomal bands were clearly evident and no RNA degradation was apparent (not shown). Following RPA analysis, aliquots (~ 500 ng) of the RNA samples were analysed to check the integrity of the samples using the Agilent Nano system. Trace readings showing the intact nature of each of the samples are shown, peaks represent the 18S and 28S ribosomal bands. A spectrum of the samples produced by the software is also shown.


a)
Lane 1
Lane 2
12 hours LPS
12 hours
4 hours LPS
4 hours
1 hour LPS
1 hour
IL-8
IL-12
GM-CSF
IL-10
IFNγ
IL-4
TGFβ
IL-6
IL-18
TNFα
IL-1β
GAPDH
IL-8
IL-12 p40
GM-CSF
IL-10
IFNγ
TGFβ
IL-4
IL-6
IL-18
TNFα
IL-1β
GAPDH
b)
1000
100
Log10 band size (nt.)
r2 0.9782
0.0
2.5
5.0
7.5
10.0
Distance migrated (cm.)
Known values
- un-protected
Un-known values
- protected

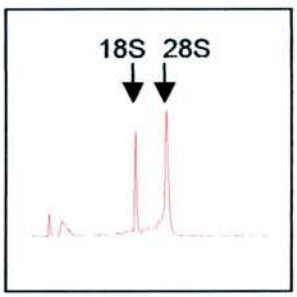
c)



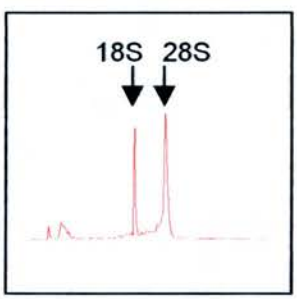
Sample 1: 1 hour



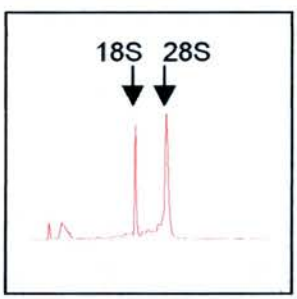
Sample 2: 1 hour LPS



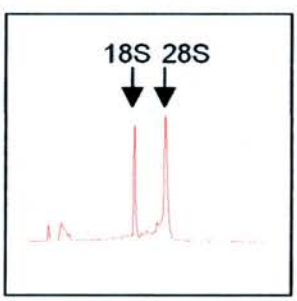
Sample 3: 4 hours



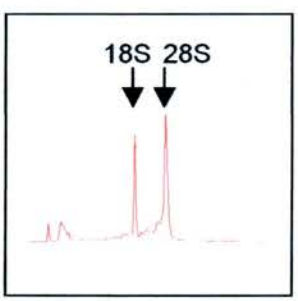
Sample 4: 4 hours LPS



Sample 5: 12 hours



Sample 6: 12 hours LPS



the RPA gel differed to the previous experiment (Fig. 4.11) in that more bands were evident. This may have been caused by degradation of the riboprobes or target samples. No degradation / increased background intensity was evident in the resolved riboprobe set (Fig. 4.26a lane 1) demonstrating that the riboprobes were intact. To check the integrity of the RNA samples analysed by RNase protection assay, aliquots of the samples were analysed following RPA analysis using the Agilent Nano system. The results are shown in Figure 4.26c, and demonstrate the intact nature of the RNA. Due to an unfortunate mishap after PAGE resolution, only the bands of higher density were analysed from samples from sheep 5, bands of lower densities were obscured as the polyacrylamide gel was not transferred in an ideal fashion for drying and exposure.

4.2.7.1 IL-18

IL-18 mRNA was detected only in cells from sheep 4; the pattern of expression is shown in Figure 4.27. LPS induced an increase in IL-18 mRNA following 1 hour culture that was 1-fold higher than medium controls, reaching peak levels after 4 hours, and decreasing following 12 hours. After 1 hour culture, control cells expressed IL-18 mRNA, which dropped below the level of detection thereafter. A comparison of the effects of LPS on IL-18 expression could only be made at the 1 hour time point (shown in Fig. 4.27), because IL-18 mRNA was not identified in control cells at the later time points. As no IL-18 message was detected in control cells, LPS induced a considerable increase in IL-18 expression during 12 hours stimulation (Fig. 4.26).

4.2.7.2 IL-12 p40

Alveolar macrophages isolated from the two sheep show similar expression kinetics and levels of IL-12 p40 mRNA (Fig. 4.28). The kinetics and levels of IL-12 p40 mRNA expression in cells purified by adherence and stimulated with LPS are similar to those in adherent BAL cells in the previous experiment (Fig. 4.15). However, IL-12 p40 expression by control cells differed markedly between experiments. Unlike in adherent BAL cells, the levels of IL-12 p40 mRNA

Figures 4.26 – 4.34 Kinetics of cytokine mRNA expression in alveolar macrophages. The expression of a panel of cytokine mRNA by alveolar macrophages cultured with LPS was assessed using an RNase protection assay (see text for details). The mean normalised value of protected radiolabelled probes for individual cytokine transcripts are plotted in x-y graphs over a time course. A comparison of mRNA levels between medium controls and LPS stimulated cells is shown; the normalised value of the mRNA transcript in each sample (LPS and medium control) are plotted against each other on a Log_{10} scale (y = LPS value, x = control value). This type of analysis shows the levels of mRNA in each sample analysed. An example is presented for IL-18 expression, the comparisons of expression of the other cytokine mRNA transcripts are in Appendix 7.

4.26 IL-18: kinetics of expression (1 – 12 hours)

4.27 IL-18: comparison of expression levels in control cells and LPS stimulated cells

4.28 IL-12 p40: kinetics of expression (1 – 12 hours)

4.29 IL-10: kinetics of expression (1 – 12 hours)

4.30 IL-6: kinetics of expression (1 – 12 hours)

4.31 GM-CSF: kinetics of expression (1 – 12 hours)

4.32 IL-8: kinetics of expression (1 – 12 hours)

4.33 IL-1β: kinetics of expression (1 – 12 hours)

4.34 TGFβ: kinetics of expression (1 – 12 hours)

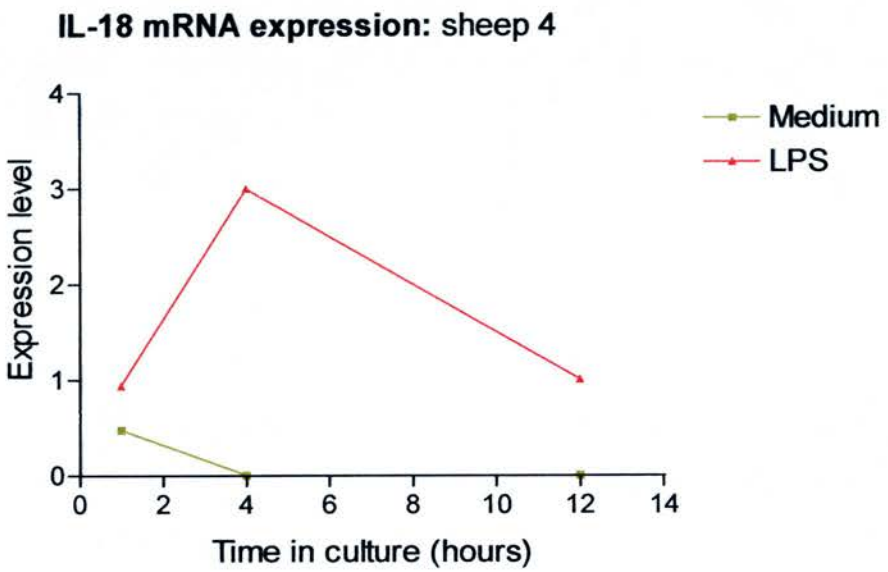


Figure 4.26 Expression of IL-18.

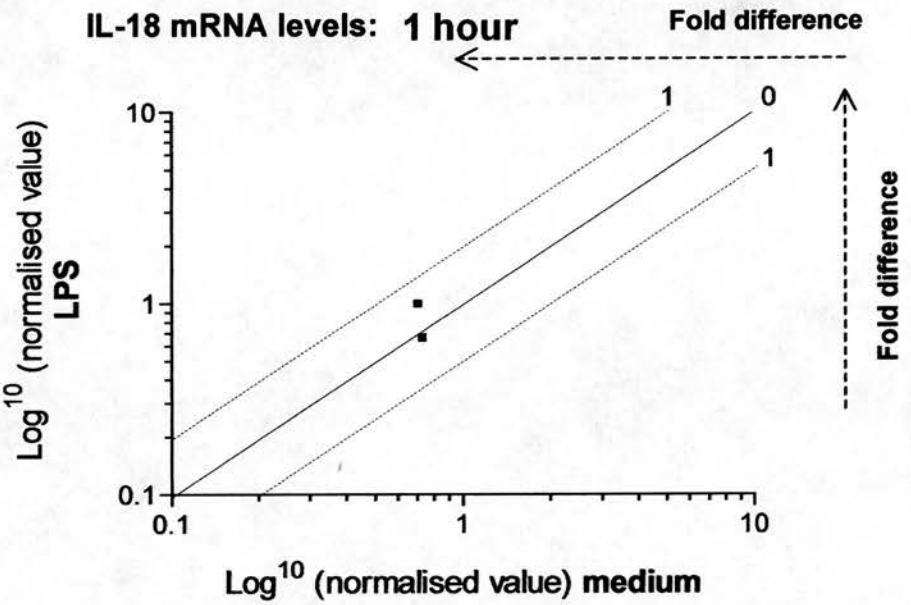
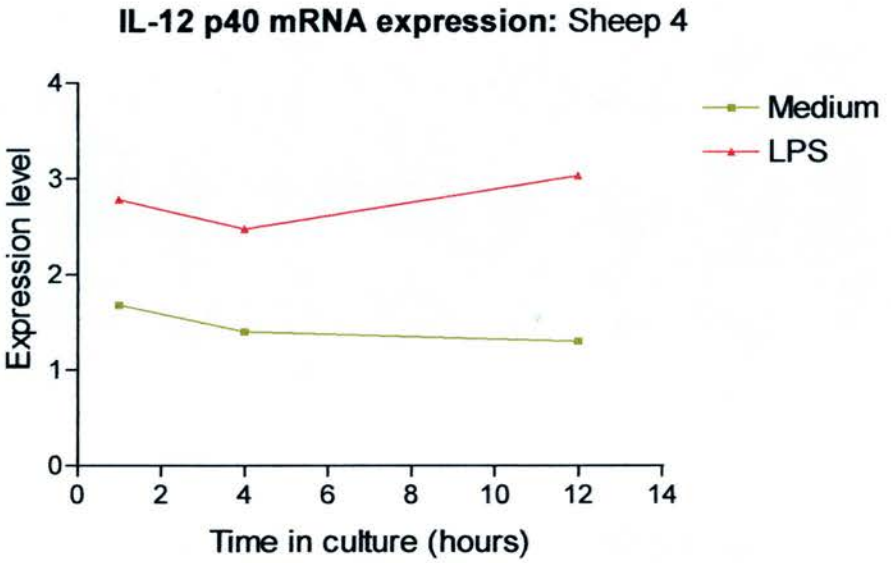


Figure 4.27 Expression of IL-18.



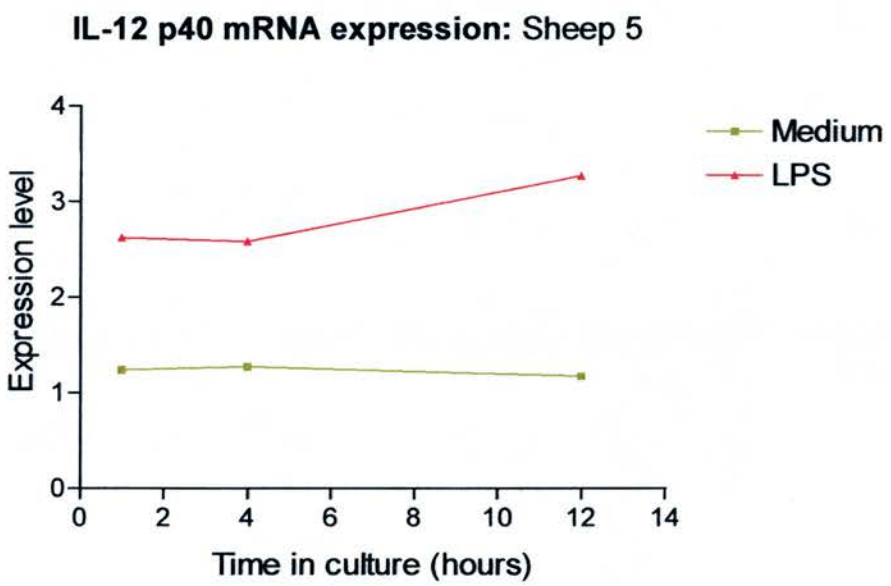
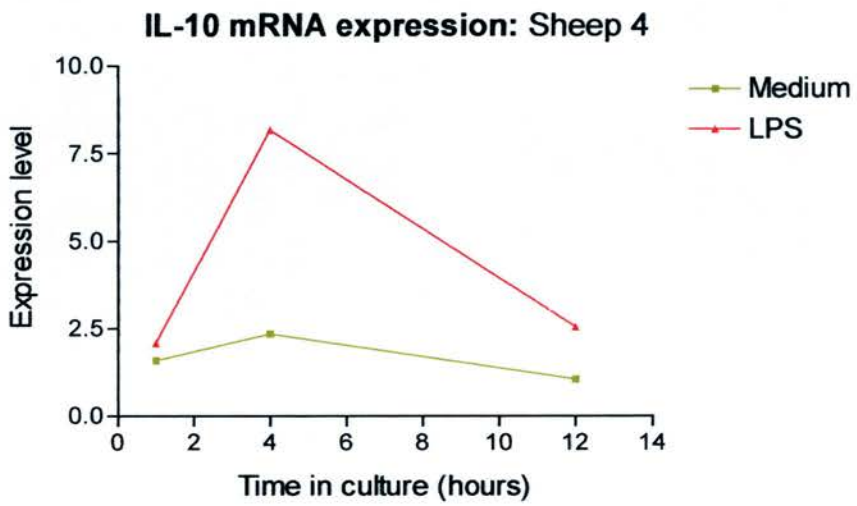


Figure 4.28 Expression of IL-12 p40.



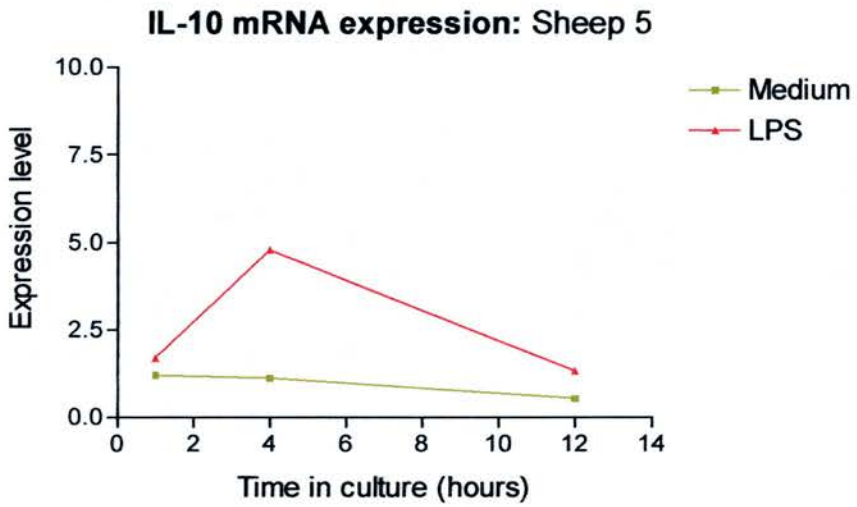


Figure 4.29 Expression of IL-10.

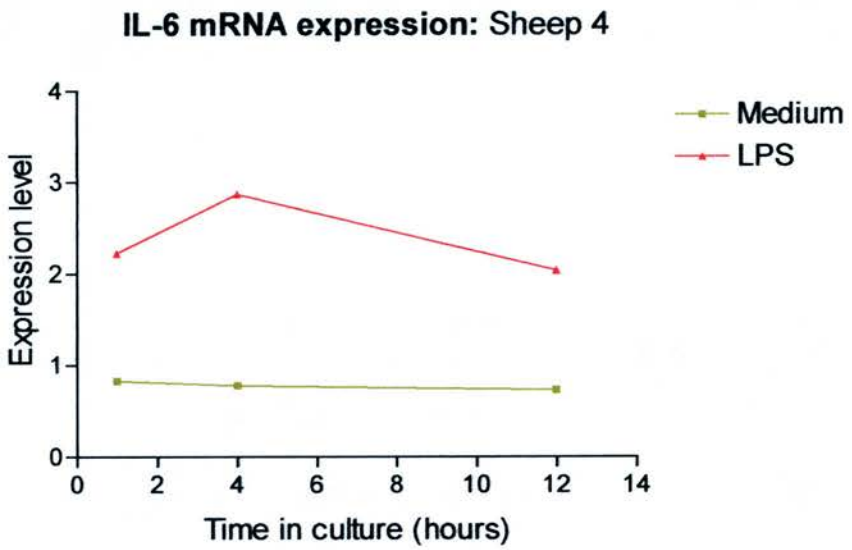


Figure 4.30 Expression of IL-6.

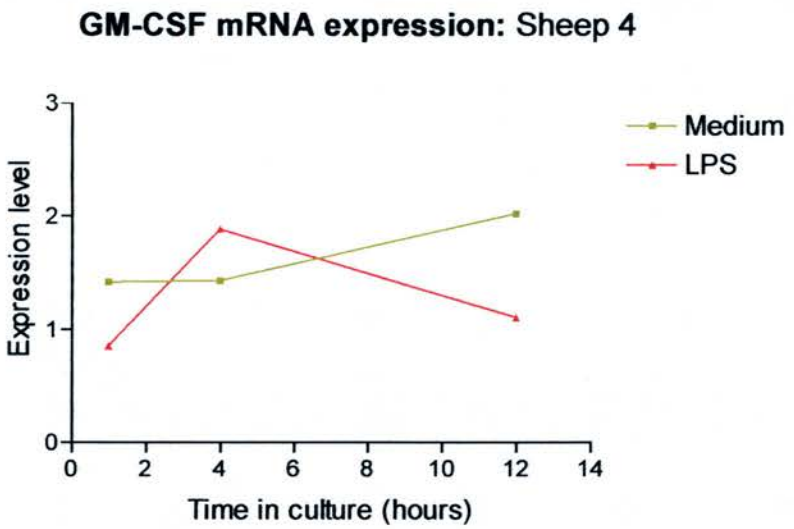
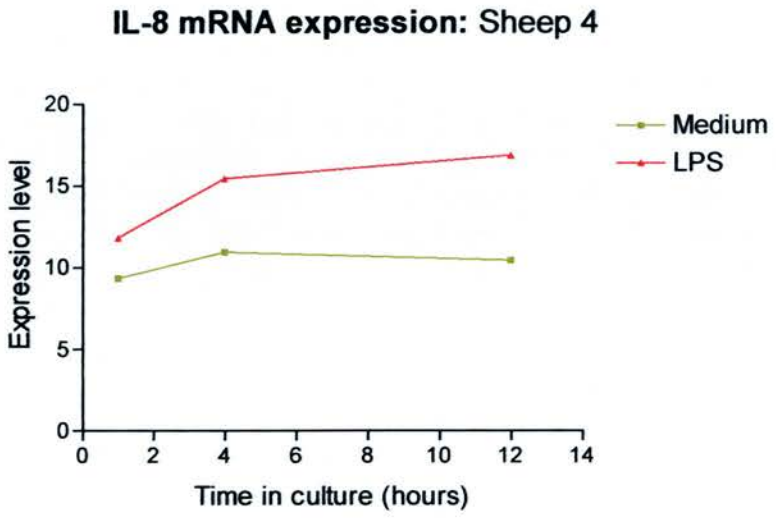


Figure 4.31 Expression of GM-CSF.



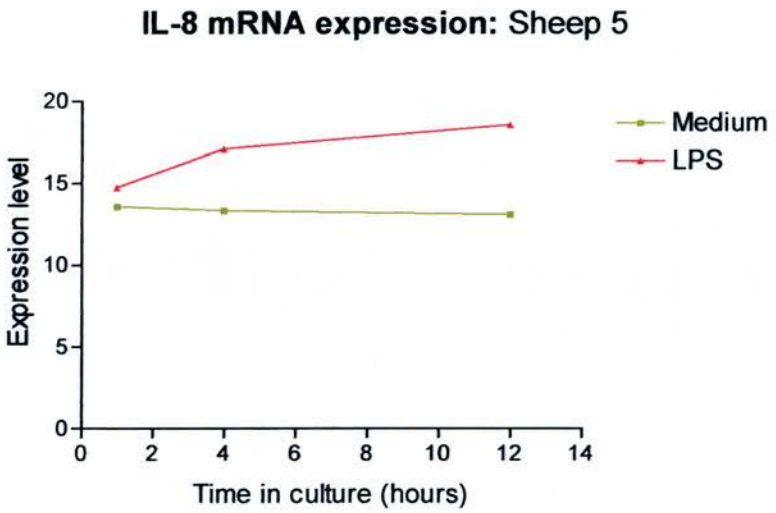
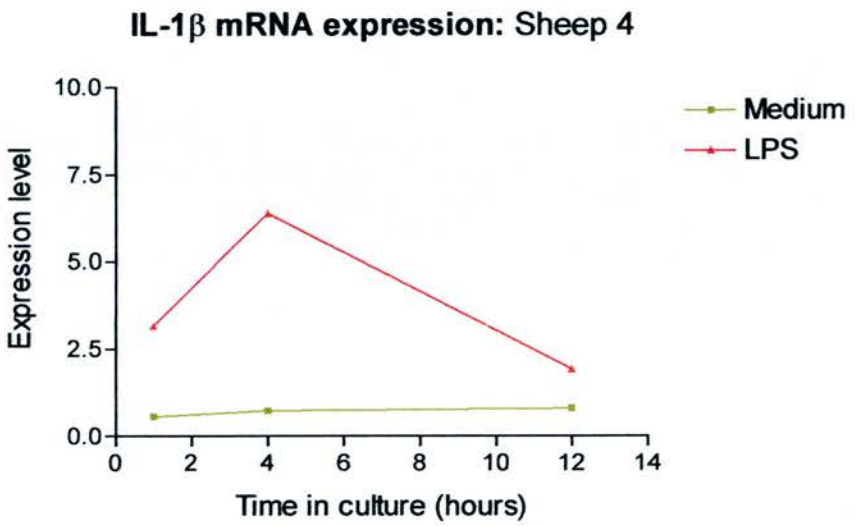


Figure 4.32 Expression of IL-8.



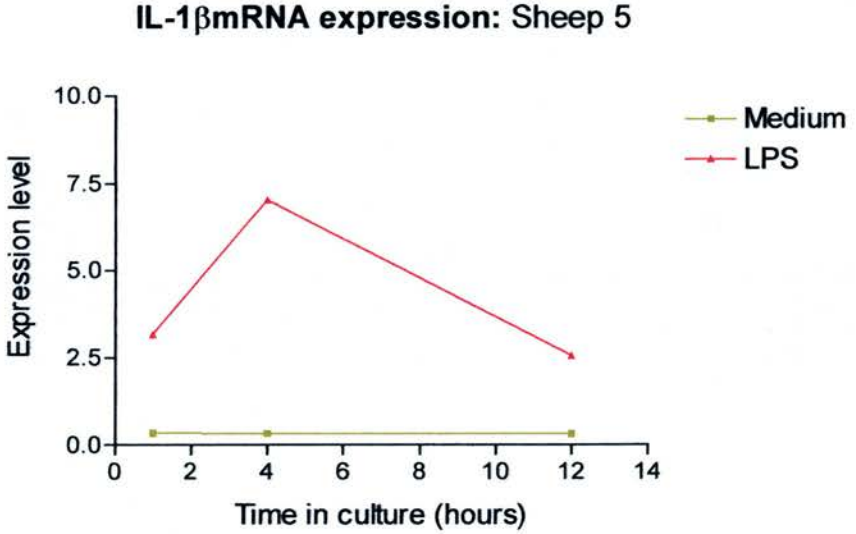


Figure 4.33 Expression of IL-1β.

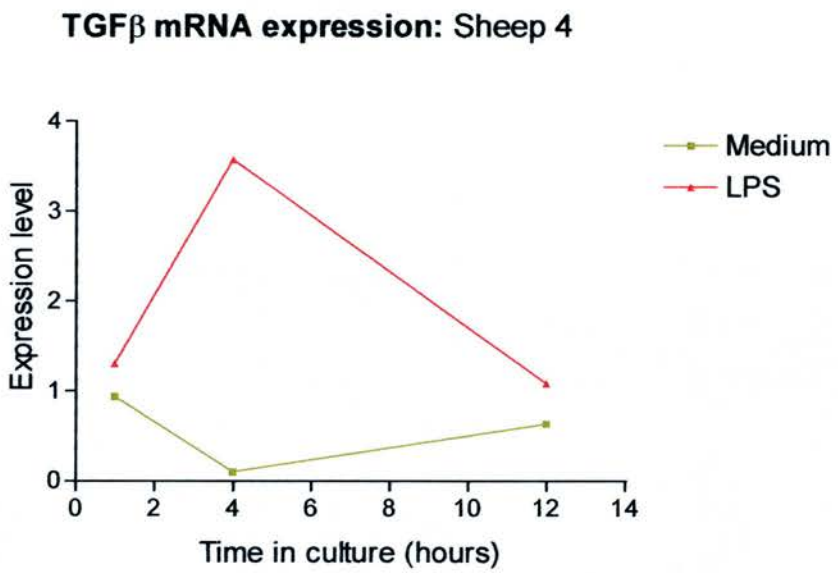


Figure 4.34 Expression of TGFβ.

expressed in purified AM cultured in medium alone remained steady, and were significantly lower than in LPS treated cells ($p \leq 0.02$) (Fig. 4.28). LPS induced enhanced IL-12 p40 expression at each time point (Appendix 7b, Fig. 1), averaging 2-fold above controls throughout the time course.

4.2.7.3 IL-10

Patterns of IL-10 mRNA expression in medium controls and LPS stimulated cells were comparable (Fig. 4.29). LPS induced a peak in IL-10 mRNA levels after 4 hours culture, the cells from sheep 4 expressing higher levels of IL-10 mRNA compared with sheep 5. IL-10 expression in controls cells remained steady over the 12 hours. AM exhibited similar patterns of LPS induced IL-10 expression when compared with BAL cells in the previous experiment (Fig. 4.16). The effects of LPS on IL-10 expression in macrophages are shown in Appendix 7b, Figure 2. LPS induced elevated IL-10 mRNA expression that was an average of 2-fold above controls.

4.2.7.4 IL-6

The expression of IL-6 mRNA during 12 hours LPS stimulation was evaluated in AM from sheep 4 (Fig. 4.30). While AM cultured in medium expressed relatively low levels of IL-6 mRNA compared with LPS stimulated cells throughout the time course, LPS induced an upwards trend in IL-6 expression following 1 hour culture, peaking after 4 hours, and dropping to levels comparable to 1 hour stimulation after 20 hours. This pattern of expression was also seen in adherent BAL cells during 4 hours and 16 hours LPS stimulation (Fig. 4.17). A prominent difference seen in AM compared with adherent BAL cells was the significant enhancement of IL-6 expression induced by LPS at each time point, averaging 3-fold higher levels than controls ($p \leq 0.02$) (Appendix 7b, Fig. 3).

4.2.7.5 GM-CSF

Figure 4.31 shows the kinetics of GM-CSF expression in cells from one donor animal (sheep 4). GM-CSF mRNA levels increased after 1 hour culture with LPS, reached peak levels after 4 hours and decreased following 12 hours stimulation.

The levels of GM-CSF mRNA in control cells increased steadily over 12 hours. Peak GM-CSF mRNA levels in AM were detected after 12 hours culture in medium alone. As shown in Appendix 7b, Figure 4; LPS had nominal effects on GM-CSF expression in AM; indeed, higher levels were detected in control cells at 1 and 20 hours culture. Whereas elevated levels of GM-CSF were expressed in LPS stimulated cells at the 4 hour time point and were moderately higher than controls (1.3-fold increase).

4.2.7.6 IL-8

The expression of IL-8 in LPS stimulated AM increased steadily over 12 hours (Fig. 4.32). The kinetics of IL-8 expression in AM were comparable to BAL cells derived from sheep 1, 2 and 3 over 4 – 16 hours (Fig. 4.19). However, the levels in BAL cells were higher than in AM. LPS had negligible effects on IL-8 mRNA levels in macrophages over 12 hours (Appendix 7b, Fig. 5)

4.2.7.7 IL-1β

AM derived from sheep 4 and sheep 5 displayed similar IL-1β expression kinetics over 16 hours in response to LPS stimulation. LPS induced an increase in IL-1β mRNA levels following 1 hour culture, increasing to peak levels after 4 hours and dropping following 12 hours stimulation (Fig. 4.33). Expression patterns and levels of IL-1β mRNA in AM in response to LPS were comparable to BAL cells over 4 – 16 hours (Fig. 4.20). LPS induced a significant increase in IL-1β mRNA over 12 hours (Appendix 7b, Figure 6). IL-1β levels were elevated 7-fold ($p \leq 0.01$) and 11-fold ($p \leq 0.01$) above controls after 1 and 4 hours IL-1β mRNA levels, respectively. This increase dropped to 4-fold higher than controls after 12 hours culture ($p \leq 0.02$).

4.2.7.8 TNFα

TNFα mRNA was not detected in AM purified by adherence and cultured with medium or LPS at any time point in either of the two sheep (Fig. 4.25).

4.2.7.9 TGFβ

The expression of TGFβ by purified alveolar macrophages stimulated with LPS is shown in Figure 4.34. Levels of TGFβ mRNA increased sharply after 1 hour culture, increasing by 3-fold to peak levels after 4 hours, and then dropping after 12 hours culture with LPS. Peak levels of TGFβ mRNA were expressed after 4 hours LPS stimulation, and were 11-fold higher than control cells at this time point ($p \leq 0.03$). At other time points, expression of TGFβ was comparable in both medium controls and LPS stimulated cells (Appendix 7, Fig. 7).

4.2.7.10 IL-4, IFNγ

The selection of AM by adherence resulted in no transcripts for IL-4 or IFNγ being detected by the RPA (Fig. 4.25).

4.2.8 The Effect of Purification by Adherence and LPS Stimulation on BAL Cell Cytokine Expression

The two methods of preparation of BAL cells for cytokine expression analysis showed marked differences in the kinetics and levels of cytokine mRNA, particularly in the control cells cultured in medium alone. Experimental data was pooled, and the level of cytokine mRNA expression induced by LPS was calculated for each time point (Table 4.4). The greatest increase in expression of the cytokines analysed was of TGFβ mRNA in purified cells after 4 hours culture (13-fold higher than basal). The effects of purifying AM by adherence were most apparent for the expression of IL-1β and TGFβ. The levels of these transcripts were considerably enhanced by LPS when compared with adherent BAL cells.

4.2.9 TNFα mRNA Detection

The RNA samples isolated from AM from sheep 4 were analysed by RT-PCR for TNFα mRNA content. Detection of GAPDH was used as an internal control for cDNA synthesis and PCR amplification. Negative controls were performed where reverse transcriptase was omitted from the cDNA synthesis reaction and template cDNA omitted from the PCR reaction; these reactions produced no product. Table

Table 4.4 Cytokine expression by ovine adherent bronchoalevolar cells and alveolar macrophages in response to 1 μg/ml LPS

Cytokine mRNA	Culture duration (hours)	Expression change relative to control		Cytokine mRNA	Culture duration (hours)	Expression change relative to control	
		aBAL	AM			aBAL	AM
IL-18	1	<2	>2	**IL-1β**	1	1	>7
	4	>2	> 4		4	<2	>10
	6	1	1		6	<1.2	-
	12 / 16	>2	> 2		12 / 16	x 0.3	>3
	20	1	-		20	<14	-
IL-12 p40	1	1	>2	**TNFα**	1	<1.5	-
	4	>2	>2		4	>2	-
	6	<1.2	-		6	1	-
	12 / 16	>3	>2		12 / 16	1	-
	20	<3	-		20	> 3	-
IL-10	1	>2	>1.5	**TGFβ**	1	<1.2	>1.4
	4	>2	>2		4	<1.5	>13
	6	>2	-		6	1	-
	12 / 16	<3	>2		12 / 16	>2.5	>2
	20	>1.3	-		20	<25	-
IL-6	1	>3	>3	**IL-4**	1	1	-
	4	>2.5	>2		4	<1.3	-
	6	>2	-		6	<1.2	-
	12 / 16	1.2	3		12 / 16	<2	-
	20	1	-		20	<8	-
GM-CSF	1	1	<1.5	**IFNγ**	1	<2	-
	4	>1.5	<2		4	1	-
	6	>2	-		6	>1.2	-
	12 / 16	>1.5	<2		12 / 16	1	-
	20	1	-		20	<3	-
IL-8	1	1	>1.2				
	4	<1.2	>1.3				
	6	1	-				
	12 / 16	>1.5	>1.5				
	20	<1.5	-				

Two experiments were performed on bronchoalveolar cells as follows; the time course began following culture (adherent bronchoalveolar cells; aBAL), or alveolar macrophages (AM) were enriched by adherence. Cytokine mRNA levels were analysed by RPA. The data from three sheep (aBAL), or two sheep (AM) were pooled and the abundance of cytokine mRNA in LPS stimulated cells was compared with medium controls. A dash (-) indicates that no data was available for that time point, > = increase, 1 = identical, < = reduction.

Table 4.5 PCR conditions for TNFα mRNA detection

Primers	Target	Cycle No.	Incubation	Extension	Taq Polymerase
AF030943F2 AF030943R2	ovGAPDH	35	94 0.5', 55 0.5', 72 0.5'	94 7'	5 units (Promega)
X55152F X55152F	ovTNFα	35	94 0.5', 55 0.5', 72 0.5'	94 7'	

RT-PCR was used to analyse TNFα mRNA expression in alveolar macrophages. Approximately 1 μg of total RNA was used as template for cDNA synthesis, and 1/10 volume used as substrate for PCR amplification. Primer sequences are listed in Appendix 4. Following analysis by agarose gel electrophoresis, PCR products were purified using a spin-bind column and cloned into pGEM-T-easy using the TA cloning system (Promega). Amplified plasmid DNA was purified using Qiagen purification spin-bind columns, and positive clones sequenced.

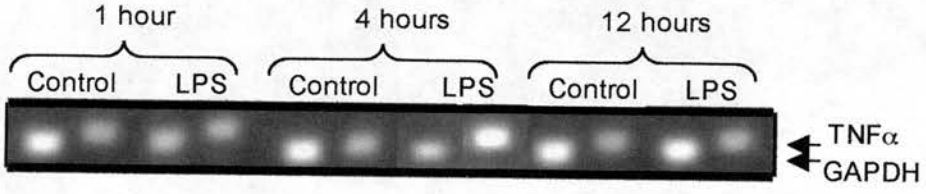


Figure 4.35 Detection of TNFα mRNA in alveolar macrophages. RT-PCR was performed on RNA isolated from control (unstimulated) and LPS stimulated (LPS) alveolar macrophages from sheep 4, as described in Table 4.6. PCR generated products of expected size: GAPDH, 104 bp; TNFα 121 bp.

4.5 shows the PCR cycling conditions used in these experiments. The results are shown in Figure 4.35. TNFα mRNA was detected in medium controls and LPS stimulated cells at each time-point (1, 4, 12 hours). The PCR product from the cDNA sample of 4 hours LPS stimulation was purified using a spin-bind column (Section 2C.4) and sequenced (MWG biotech). Analysis using GAP software (GCG 10) revealed 100% homology to sheep TNFα (X55152), and the reverse, complementary sequence of the TNFα riboprobe (not shown).

4.3 DISCUSSION

4.3.1 RPA Development

The quantification of RNA transcripts by RPA relies on the linear relationship between band intensity and the concentration of target RNA species in the starting material. Increasing amounts of cellular RNA were hybridised with complementary riboprobes (GAPDH, IL-1β, IL-10, IL-8 and IL-18) in a probe set (Section 4.2.2.1). A direct correlation between band intensity and amount of hybridised RNA was demonstrated for the detection of GAPDH, IL-1β, IL-10 and IL-18. The exception was IL-8, that showed a non-linear relationship to GAPDH intensity (Section 4.2.2.1). The phosphorimage scan for this particular experiment showed that the IL-8 probe signal was present at high levels, and peak thresholds on the phosphorimage scan were met. It seems, therefore, for exceedingly high levels of target RNA (such as IL-8 mRNA), the sensitivity of the RPA was compromised. IL-8 was the most highly expressed cytokine in all experiments.

The sensitivity of the RPA for the measurement of transcripts expressed at low levels was also a consideration. The riboprobes were labelled with [^{32}P] which allows the detection of small quantities of mRNA. Direct labelling of mRNA targets expressed at low levels may be below the detection levels of this assay. For example, it was demonstrated that IL-18 was not detected by RPA analysis of a 10 μg sample of LPS stimulated macrophage RNA. However, IL-18 was demonstrated in the RNA sample by RT-PCR (Section 4.2.2.2). As a positive control the use of complementary RNA showed that 1 pg of plasmid expressed IL-18 mRNA was detected by the RPA.

The primary function of alveolar macrophages *in vivo* seems to be the uptake and neutralisation of environmental antigen. Although AM are considered the end stage development of monocytes, they exhibit great functional activity related to inflammation and antimicrobicidal activity such as the release of IL-1β (Nash et al., 1992; Foss, Zilliox and Murtaugh, 1999), IL-6 (Andrews et al., 1993; Assoian et al., 1987) and chemokines (Kopydlowski et al., 1999). Ovine AM cytokine expression profiles determined by the RPA should reflect the inherent function of these cells.

The data showed that:

- sheep alveolar macrophages constitutively expressed IL-8, IL-1β, IL-18 and TGFβ transcripts
- the immediate affects of culturing bronchoalveolar lavage cells (BAL) induced peak expression levels of many cytokines
- in the absence of alveolar macrophages enrichment, cytokine expression by the adherent cell population cultured in medium correlated with LPS stimulation
- LPS induces the expression of cytokines in AM that have roles in innate and adaptive immunity

Each of these findings is discussed below.

4.3.2 Measurement of Cytokine Expression in Resting cells

Freshly isolated AM derived from one sheep expressed IL-8, IL-1β, IL-18 and TGFβ. BAL cells were enriched for macrophages using a density media separation protocol (Section 2A.3). It should be noted that these data were derived from a single experiment involving one animal, which was not repeated due to time-constraints of the project. Detection of IL-18 mRNA in the resting AM indicated that IL-18 mRNA abundance was greater than 1 pg / 10 μg of total cellular RNA. IL-18 is postulated to have a relatively long half-life compared to other cytokines, due to a lack of destabilising elements in the UTR (Tone et al., 1997). It is regulated at the post-transcriptional level by the expression of caspase-1 that is required for the secretion of the bioactive protein. These results indicate that similar to macrophages isolated from different antatomical locations (murine peritoneal exudates and liver) (Okamura et al., 1995b; Puren, Fantuzzi and Dinarello, 1999), IL-18 is expressed constitutively in sheep alveolar macrophages.

In the healthy lung, constitutive cytokine expression has been documented which is thought to contribute to immune regulatory and homeostatic mechanisms. TGFβ has been found to be constitutively expressed in human AM (Assoian et al., 1987) and in lymph draining sheep lungs (Perkett et al., 1990). Its role may be associated with suppressor functions limiting T cell activation in epithelia and lung mucosa. TGFβ may also support cell homeostasis and repair (Yamauchi et al.,

1988). Constitutive expression of IL-18 has been documented in rat lungs (Brieland et al., 2000; Jordan et al., 2001), and freshly isolated AM express IL-8 (Strieter et al., 1990) and IL-1 (Koretzky et al., 1983; Lamontagne et al., 1985). IL-8 is a potent chemoattractant for many leucocytes. IL-1β and IL-18 enhance neutrophil recruitment to tissue through enhanced expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Jordan et al., 2001; Karmann et al., 1996). The constitutive production of IL-8, IL-1β and IL-18 in the lung may provide a 'chemoattractant reservoir' supporting the recruitment of circulating phagocytes and lymphocytes.

4.3.3 Measurement of Cytokine Expression by Adherent BAL cells

Independent studies have reported that LPS induces a significant increase in IL-1β, IL-6 and TNFα mRNA levels in AM compared to controls, with peak levels observed 3-6 hours after stimulation (Andrews et al., 1993; Nash et al., 1992). In the current study, there were two unexpected trends in cytokine expression of BAL cells. First, peak levels of IL-1β, IL-6, IL-8, IL-12 p40, IL-18, GM-CSF, TGFβ and TNFα mRNA transcripts were found after 1 hour culture in AM from at least one of the sheep. Primary cells require time to acclimatise to culture conditions and increased cytokine expression by the BAL cells may in part be attributed to immediate responses to culture. An important consideration is the effects of cell adherence to the tissue culture flask. Attachment induces human monocyte / macrophage expression of IL-1β, TNFα and IL-6 (Brodbeck et al., 2002), and indeed peak levels were seen for these transcripts in sheep adherent BAL cells after 1 hour culture. Second, the levels of cytokine transcripts in medium controls were similar to cells cultured with LPS. Moreover, IFNγ and IL-4 mRNA were detected in both control cells and LPS stimulated cells. IFNγ is primarily expressed by activated T cells and NK cells, but has also been documented to be produced by human AM stimulated with LPS, phytohaemagglutanin (PHA), poly I:C (Nugent et al., 1985) or IL-12 (Fenton et al., 1997) and in lung macrophages from mice in response to *Mycobacterium bovis* bacillus Calmette-Guerin plus IL-12 (Fenton et al., 1997). IL-4, however, is produced by activated T cells and mast cells (Brown et al., 1987) including lung mast cells (Gibbs et al., 1997); macrophage production of IL-4 has not

been reported. The detection of IL-4 indicates that lymphocytes and / or mast cells were present in the adherent BAL cells. In addition to contributing to the pool of RNA analysed by the RPA, the presence of lymphocytes and mast cells in the cultures may have influenced the responses of the adherent AM. In the absence of LPS, inducible expression of IL-1β, IL-6 IL-12, IL-10 and TNFα, has been documented following CD40-triggering of macrophages and DCs, and has been shown to be dependent on IFNγ and IL-4 (Maus et al., 1998; Nagayama et al., 2000; Snijders et al., 1998; Hochrein et al., 2000; Kalinski et al., 2000; Ebner et al., 2001). CD40L is expressed by CD8 T cells, eosinophils, mast cells, NK cells, but primarily by activated CD4 T cells, and CD40 is expressed on B cells, DCs and activated macrophages. CD40 ligation on macrophages or DCs induces an up-regulation of the production of IL-1β, TNFα and IL-12 (Kato et al., 1996; Koch et al., 1996; Yang and Wilson, 1996; Grewal et al., 1996; Stout and Suttles, 1996; Morelli et al., 2001). AM in mice express low levels of CD40 (Zhang Hoover, Sutton and Stein Streilein, 2001) and whether this is true in sheep is not yet known. The indication of activated lymphocytes provides a mechanism of CD40 triggering and the demonstration of IL-4 and IFNγ could contribute to increased cytokine production in the adherent BAL medium control cultures.

4.3.4 Measurement of Cytokine Expression by AM Stimulated with LPS

In review of the data and the conclusions drawn above, a second series of experiments were performed by purifying AM by adherence (Section 4.2.7). LPS significantly enhanced expression of IL-18 (4-12 hours), IL-12 p40 (1–12 hours), IL-1β (1-12 hours), IL-6 (1-12 hours) and TGFβ (4 hours). IL-18 and IL-12 p40 play prominent roles in inducing IFNγ production by NK cells and T cells, and the development of antigen-specific cell mediated immunity (Th1-type responses) (Magram et al., 1996; Fantuzzi et al., 2000). LPS induction of IL-18 and IL-12 p40 contributes to protective immunity in both the early and late phases of the response. In addition to IFNγ production, IL-18 induces the synthesis of IL-1β and TNFα and IL-8 (Puren et al., 1998), which contribute to the recruitment of phagocytes.

During responses to intracellular infections affecting the lung, there is enhanced production of IL-10 (Jacobs et al., 2000; Huang et al., 1999) and IL-6 (van

der Poll et al., 1997). IL-10 is usually considered as regulatory and anti-inflammatory. Production of IL-1β, TNFα, IL-6 and IL-8 by AM can be inhibited by IL-10 (Thomassen, Divis and Fisher, 1996). IL-6 is a pleitrophic cytokine that can be considered has having pro-inflammatory and anti-inflammatory properties. IL-6 has been demonstrated to be particularly important in regulating lung inflammatory responses *in vivo* (Barton and Jackson, 1993; Xing et al., 1998; Ward et al., 2000).

Thus, under the influence of culture and LPS stimulation, ovine AM produced both pro-and anti-inflammatory cytokines, which may help maintain immunological equilibrium *in vivo*.

4.3.5 Cytokine mRNA Expression Kinetics

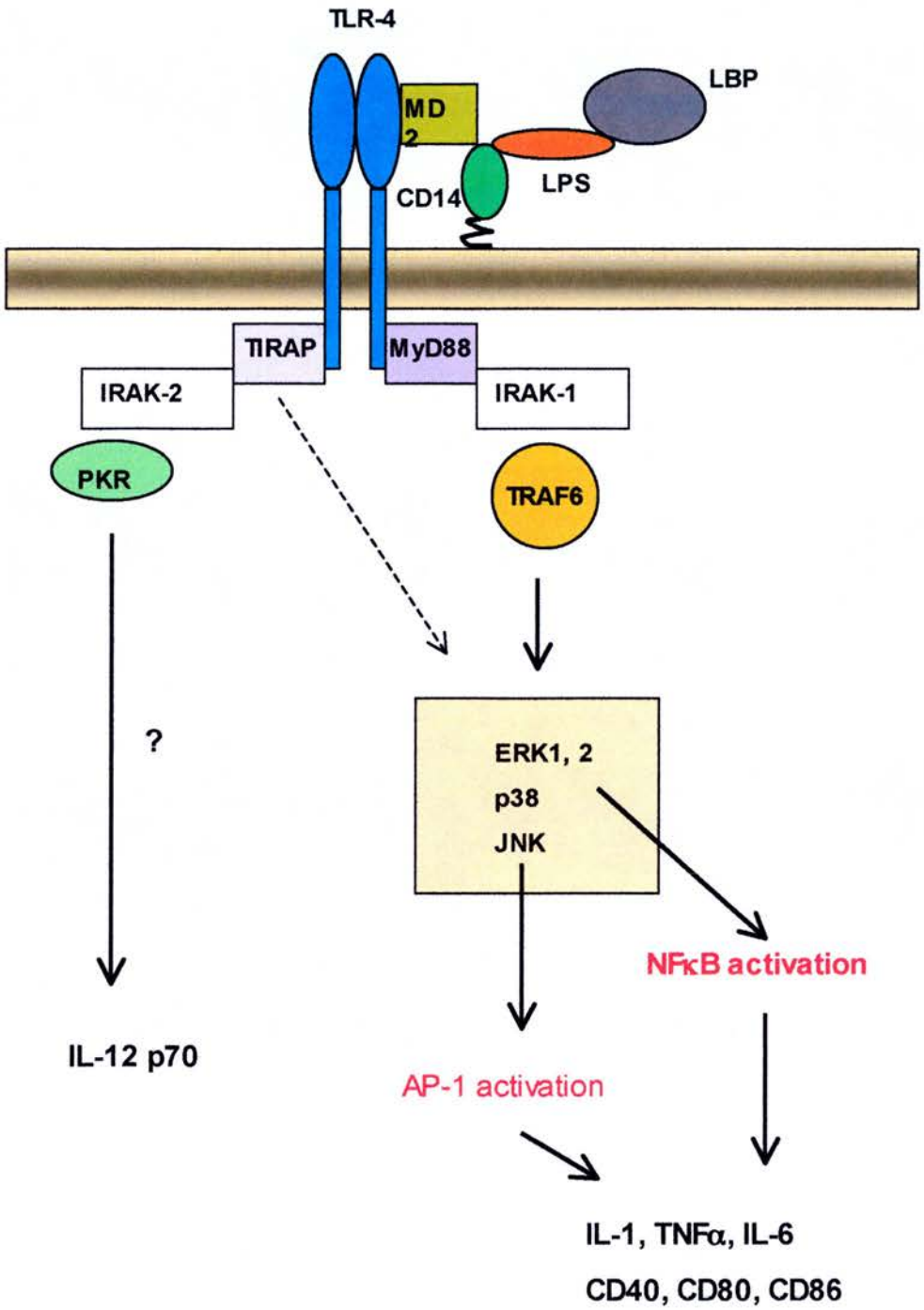
The expression of IL-18 in AM showed similar kinetics to IL-1β, IL-6, IL-10, TGFβ and GM-CSF in response to LPS. After treatment with LPS the amount of specific RNA increased according to the levels measured in controls, levels peaked after 4 hours and decreased thereafter. GM-CSF mRNA was moderately enhanced by LPS compared to controls (Section 4.2.7.5), this has also been reported for murine peritoneal exudate macrophages that seem to produce GM-CSF mRNA in response to FCS in the culture medium (Thorens, Mermod and Vassalli, 1987). Increased release of GM-CSF seems to be controlled by post-transcriptional mechanisms (Thorens, Mermod and Vassalli, 1987), so measuring mRNA transcription levels may not be an accurate way to quantify GM-CSF production.

LPS signals through CD14 and a transducing receptor such as toll-like receptor-4 (TLR4) (Poltorak et al., 1998; Qureshi, 1999). A schematic of the signalling response of LPS is presented in Figure 4.36. Signalling through toll-like receptors requires the adaptor protein MyD88 (Kawai et al., 1999; Adachi et al., 1998). MyD88 functions to recruit down stream signalling molecules to the receptor complex, such as IL-1R-associated kinase (IRAK)-1 (Wesche et al., 1997). Upon recruitment to the receptor complex, IRAK-1 is autophosphorylated and associates with TRAF-6, leading to the activation of the MAP kinase (MAPK) pathways, ERK 1, 2 and 3 (p38) and c-Jun N-terminal kinase (JNK). Activation of MAPK pathways leads to phosphorylation of IκB, dissociation of the IκB complex and the

translocation of nuclear factor-kappa B (NFκB), and activation of activator protein-1 (AP-1). The activation of the transcription factors and NFkB and AP-1 culminates in altered expression of a multiple genes. An additional adaptor signalling molecule of TLRs is TIR domain-containing adapter protein (TIRAP) (Horng, Barton and Medzhitov, 2001) that is also capable of regulating signals culminating in p38 and NFkB activation as it can interact with MyD88. Independently of this pathway, TIRAP can associate with IRAK2 and protein kinase receptor (PKR) driving the production of IL-12 p40 (Fitzgerald, 2001; Horng, Barton and Medzhitov, 2001; Kawai et al., 2001).

The IL-18, IL-1β, IL-6, TGFβ and GM-CSF genes contain NFκB targets in their enhancer regions (Droogmans et al., 1992; Hiscott et al., 1993; Guron, Sudarshan and Raghow, 1995; Tone et al., 1997; Cruz et al., 2001), and IL-10 expression is activated by the p38 MAPK pathway (Ma et al., 2001). A common pathway of gene activation could explain the simultaneous expression kinetics of IL-18, IL-1β, IL-6, IL-10, TGFβ and GM-CSF soon after LPS treatment in the current study. With the exception of IL-18 (Tone et al., 1997), these mRNAs are rapidly induced with a relatively short half-life (Carter and Malter, 1991), so their immediate levels are a good reflection of gene induction. The early peak of cytokine transcript expression in response to LPS presented here demonstrates the production of cytokines with convergent roles in the innate protective response to gram negative bacterial infection, and supports published data regarding the rapid activation of endogenous signalling pathways in response to LPS. Murine liver and peritoneal macrophages exhibit maximal activation of NFκB 1 hour after LPS treatment, but decline after 3-6 hours (Velasco et al., 1997). During stimulation with LPS, TLR4 levels gradually decrease and are dramatically reduced following 24-hours pre-treatment (Nomura et al., 2000), providing a mechanism for the down-regulation of LPS-induced genes. In addition, Li et al. (2000) reported that a decrease in IRAK levels are seen after 1-3 hours LPS stimulation of THP-1 cells. LPS-induced MyD88-IRAK interaction persist until 1 hour after LPS stimulation. From the results

Figure 4.36 Toll-like receptor 4 signalling pathway. The Toll-like receptor (TLR) utilises common signalling molecules such as the MyD88 adapter protein, the protein kinase IRAK (IL-1R-associated kinase) and TRAF6 (TNF receptor-associated factor 6). TRAF6 activates nuclear factor-κ B (NFκB) and the MAP kinase (MAPK) signalling pathways, p38 and c-Jun N-terminal kinase (JNK). Another adapter molecule, TIRAP (TIR domain-containing adapter protein) interacts with MyD88 and induces activation of NFκB. TIRAP interacts with protein kinase receptor (PKR) inducing activation of as yet un known signalling pathways that are MAPK-independent and culminate in the induction of IL-12 p40 promoter activity. (Figure adapted from reviews; (Medzhitov, 2001; Jefferies & O'Neill, 2002; O' Neill, 2002)


TLR-4
MD 2
LBP
LPS
CD14
TIRAP
MyD88
IRAK-2
IRAK-1
PKR
TRAF6
?
ERK1, 2
p38
JNK
NFκB activation
IL-12 p70
AP-1 activation
IL-1, TNFα, IL-6
CD40, CD80, CD86

using the RPA presented in this chapter, a down-regulation of NFκB-activated genes (IL-18, IL-1β, IL-6, IL-10, TGFβ and GM-CSF) is evident after 4 hours stimulation. This supports published data of the kinetics of NFκB activation following LPS treatment (Velasco et al., 1997; Nomura et al., 2000; Li et al., 2000), and the IL-18 inducible promoter that exhibits maximal activation after 3-6 hours LPS treatment (Kim et al., 1999).

4.3.6.1 IL-12 p40 and IL-8

The expression kinetics of IL-12 p40 and IL-8 differed from the other cytokines. There was a gradual increase in IL-12p40 mRNA levels during the 12 hour LPS time-course (Section 4.2.7.2). Although NFκB and AP-1 drive IL-12 p40 gene expression (Grumont et al., 2001; Zhu et al., 2001), during IL-12 p70 synthesis these transcription factors are activated by a MAPK-independent pathway involving PKR (O' Neill, 2002). The data indicate that the regulation of IL-12 p40 differs to other immediate response genes (IL-18, IL-1β, IL-6, IL-10, TGFβ and GM-CSF) in LPS stimulated AM. The expression of IL-8 showed a gradual increase during the 12-hour time-course, and in addition, IL-8 mRNA levels in medium controls were not significantly different to LPS treated cells (Section 4.2.7.6). The non-linear detection of IL-8 transcripts using the RPA showed that quantification using the method as currently optimised is un-reliable. Therefore further optimisation of the RPA for IL-8 transcript quantification is necessary. The kinetics of IL-8 mRNA expression by ovine AM stimulated with LPS were similar to that reported for porcine AM (Lin et al., 1994). Lin et al. (1994) also demonstrated that cultured AM express detectable levels of IL-8 mRNA, and that LPS induces a significant increase (4 fold) in specific mRNA. A possible explanation for the statistically non-significant increase in IL-8 mRNA in response to LPS reported here is that peak thresholds were met, inhibiting the concurrent measurement of IL-8 message.

4.3.7 TNFα mRNA Detection

LPS induction of high levels of TNFα mRNA have been documented in AM recovered from humans and sheep (Becker, Devlin and Haskill, 1989) (Nash et al., 1992), thus non-detectable levels of TNFα mRNA using the RPA in both sets of

experiments were unexpected. The efficiency of hybridisation of each riboprobe to complementary RNA synthesised from plasmid DNA was demonstrated in each experiment as a positive control, and the TNFα product was evident (not shown). To determine the presence of 'target' mRNA in the cells, RNA samples were analysed by RT-PCR, using primers within the riboprobe region, and TNFα mRNA was detected (Section 4.2.9). It was possible that the TNFα riboprobe contained regions of ambiguity for its target, so the PCR products were sequenced. The TNFα message detected by RT-PCR was 100% complementary to the corresponding riboprobe. It is possible that hybridisation conditions for this particular probe were not optimal resulting in low hybridisation stringency and very low levels of detection.

Out of the eleven cytokine probes tested, TNFα was not optimally functional using this protocol.

4.3.8 Summary

The development of the RPA and its subsequent application to the measurement of cytokine mRNA levels in alveolar macrophages was used as a means of investigating the RPA quantitative nature and potential uses. Overall, the the data indidcated that the RPA was developed successfully for the measurement of the sheep IL-1β, IL-4, IL-6, IL-10, IL-12 p40, IL-18, GM-CSF, IFNγ, TGFβ with the exception of TNFα, and possibly IL-8.

For the measurement of mRNA transcripts, the RPA displayed a sensitivity of detecting ~1 pg of RNA in the target sample. To enable the detection of low level transcripts, such as IL-18, at least 15 μg of macrophage RNA was required.

The data show that the determination of transcript abundance in replicate RNA samples exhibited high reproducibility as demonstrated in the scatter graphs displaying the relative ratio of mRNA levels at each time-point (Appendix 7).

In addition, the kinetics of expression of cytokine mRNA in response to LPS stimulation were in agreement with published data for IL-1β (Nash et al., 1992; Foss, Zilliox and Murtaugh, 1999), IL-6 (Andrews et al., 1993), IL-12 p40 (D'Andrea et al., 1992; Marshall et al., 1999), IL-18 (Marshall et al., 1999), TGFβ (Assoian et al., 1987) and GM-CSF (Thorens, Mermod and Vassalli, 1987) expression by

macrophages or peripheral blood monocytes. These data help validate the specificity and measurement of ovine cytokine mRNA using the RPA.

Chapter 5: Characterisation of Ovine Lymph Dendritic Cells

5.1 INTRODUCTION

5.1.1 Lymph DCs: Function and Sub-types

Ovine afferent lymph DCs stimulate strong allogeneic responses in resting T cells, and moreover induce strong proliferative responses in T cell lines to antigen collected *in vivo* (Bujdoso et al., 1989). These T-stimulatory characteristics have also been documented for cattle lymph DCs (McKeever et al., 1991), and rat lymph DCs draining the gut (MacPherson and Liu, 1993).

Two populations of cattle lymph DCs have been characterised based on the expression of the signal inhibitory receptor-α molecule (SIRPα; originally named MyD-1) (Howard et al., 1997). The major population, SIRPα$^+$ DCs, was more proficient at stimulating allogeneic CD4 and CD8-responses than the SIRPα$^-$ DC population at a range of ratios of DC:responder cells. Stimulation of antigen specific responses also differed between the DC populations, at high DC:responder ratio cell (1:10), SIRPα$^-$ DCs induced a stronger proliferative response in memory CD4 T cells, whereas at lower ratios (1:100-1000; DC:CD4 T cells) SIRPα$^+$ DCs induced slightly stronger antigen specific responses (Howard et al., 1997). It appeared that the differences in the capacity for cattle lymph SIRPα DC populations to induce CD8$^+$ allogeneic responses was due to differential cytokine production, and may be dependant on IL-1α secretion by SIRPα$^+$ DCs (Hope et al., 2001).

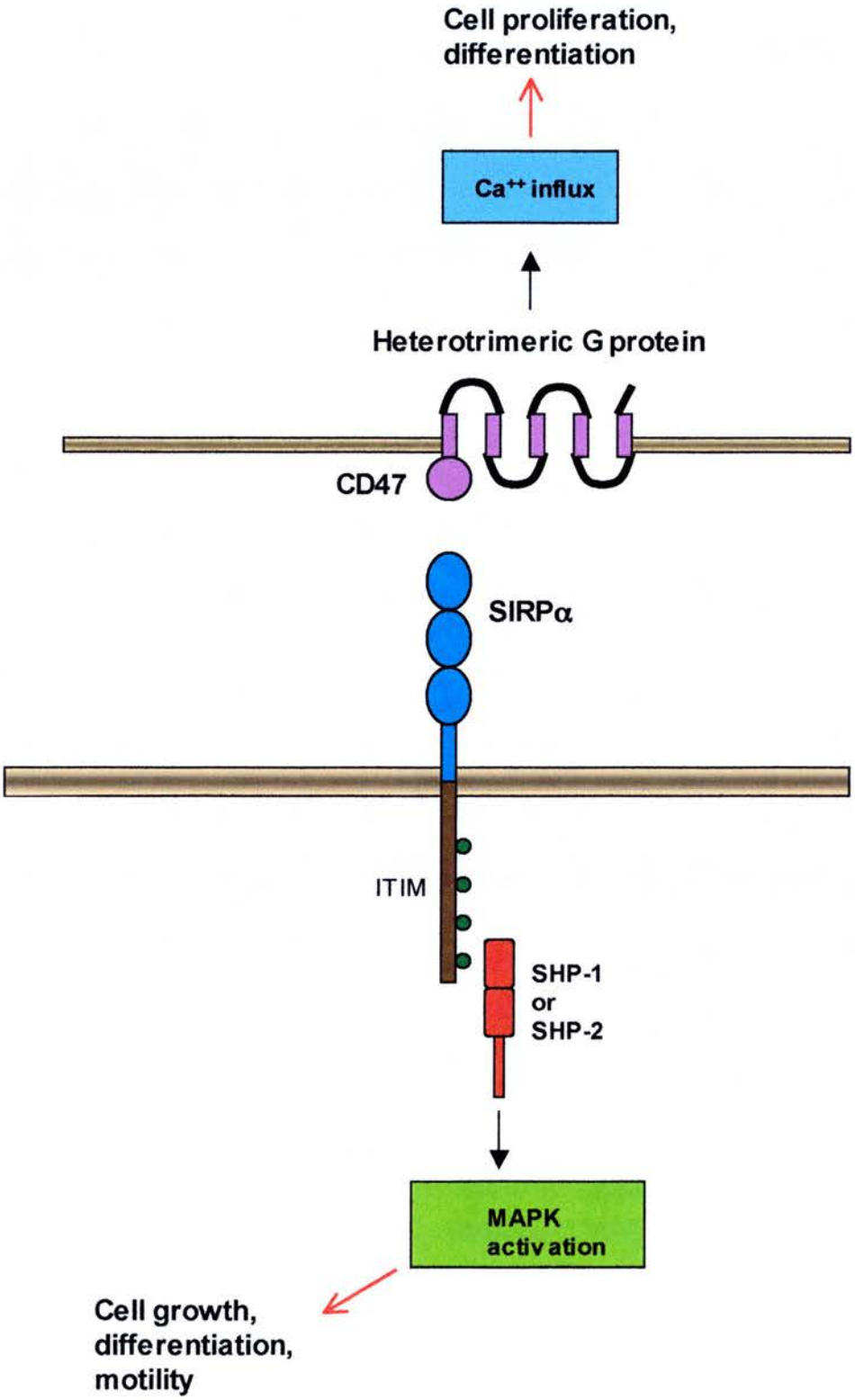
SIRPα DC populations have also been described in rat mesenteric lymph (recognised by the mAb OX41 [Adams et al., 1998]). Freshly isolated rat SIRPα$^+$ DCs were markedly more efficient at stimulating antigen-specific memory T cell responses, and allogeneic responses at a range of DC:responder cell ratios (Liu et al., 1998). Therefore, at least functionally, bovine and rat lymph SIRPα DC populations appear to be similar.

5.1.2 Signal Regulatory Protein-α

SIRPα contains a cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM), whereas SIRPβ lacks an intracellular portion and its function is unclear (Kharitonenkov et al., 1997). SIRP proteins have been designated signal regulatory proteins, as it seems that SIRPα sequesters intracellular src-homology-1-domain containing protein tyrosine phosphatases (SHP)-1 and SHP-2 away from protein tyrosine kinases (PTKs) (Fujioka et al., 1996; Kharitonenkov et al., 1997; Veillette, Thibaudeau and Latour, 1998). PTKs are involved in antigen-receptor signalling and the positive and negative regulation of cell growth and differentiation, chemotaxis and actin re-organisation (Hubbard and Till, 2000). Thus SIRPα has a potential role regulating a variety of cellular functions.

SIRPα mRNA is ubiquitously expressed in most tissues in humans (heart, brain, spleen, lung, liver, muscles, kidney and testis), being most abundant in brain and spleen (Kharitonenkov et al., 1997). Using immunohistochemistry it has been shown that SIRPα is strongly expressed on rodent myeloid cells (macrophages, monocytes, granulocytes, DCs) and neurons (Adams et al., 1998). DC expression of SIRPα has been demonstrated in a number of mammals including rodents, cattle and humans, (Adams et al., 1998; Brooke, Parsons and Howard, 1998; Seiffert et al., 1999; Dietrich et al., 2000; Huang et al., 2000; Hope et al., 2001; Latour et al., 2001). Interactions with SIRPα and its ligand CD47 (integrin-associated protein) on T cells seem to enhance cell-cell adhesion (Seiffert et al., 1999). A schematic representation of SIRPα and CD47 signalling pathways is presented in Figure 5.1. SIRPα and CD47 interactions result in 'bi-directional' signalling, activating a variety of responses in the participating cells. Latour and colleagues (2001) demonstrated that incubation of human MoDCs with an SIRPα antagonist (CD47Fc), suppressed the secretion of IL-12, TNFα, IL-6 and IL-10 in response to bacterial products (Latour et al., 2001). In addition, DC maturation was also suppressed, as measured by the expression of CD40, CD80 and CD86 (Latour et al., 2001). CD47 engagement on T cells down-regulated IL-12 receptor expression, and MoDCs incubated with CD47Fc

Figure 5.1 Signal regulatory protein-α and its ligand CD47: intracellular pathways of activation. Activation of signal protein-a (SIRPα), such as during interaction with its natural ligand CD47, induces phosphorylation of its cytoplasmic domain-tyrosines creating an immunoreceptor tyrosine-based inhibition motif (ITIM). The activated ITIM recruits Src-homology 2 (SH2)-domain containing tyrosine phosphatases, SHP-1 and SHP-2. SHP-1 and SHP-2 regulate the MAP kinase (MAPK) signalling pathways, which are involved in the activation of cellular processes, such as growth and differentiation. Interaction of the extracellular region of SIRPα with CD47 transduces 'bi-directional' signalling. Ligation of CD47 activates G protein-mediated signalling pathways including an increase in intracellular calcium, culminating in cell proliferation and differentiation. (Figure adapted from review [Oshima, 2002]).


Cell proliferation, differentiation
Ca++ influx
Heterotrimeric G protein
CD47
SIRPα
ITIM
SHP-1 or SHP-2
MAPK activation
Cell growth, differentiation, motility

were markedly less efficient at inducing IFNγ production in an allogenic MLR than controls (Latour et al., 2001). These data suggest that during DC-T cell contact, CD47-SIRPα interactions play a regulatory role in the production of inflammatory cytokines and the development of Th1-type responses. Interestingly, as mentioned previously SIRPα$^+$ lymph DCs were more efficient at stimulating allogeneic and autologous T cells than the SIRPα$^-$ cells (McKeever et al., 1991; Howard et al., 1997; Liu et al., 1998). A SIRPα-specific mAb, IL-A24, blocked T cells binding to SIRPα (MyD-1) transfected COS-7 cells and abrogated monocyte induced proliferation of T cells (Ellis et al., 1988; Brooke, Parsons and Howard, 1998). However, the addition of saturating concentrations of purified IL-A24 to DC co-cultures with a T cell clone did not seem to have any effect on antigen induced T cell proliferation (McKeever et al., 1991). It seems therefore, that SIRPα may have differential functions depending on the cell type on which it is expressed, and the activation state or activity of the cell.

5.1.3 Chapter Aims

Whether the SIRPα DC populations exist in ovine afferent lymph was not known, and was the initial aim of these sets of experiments. This chapter will describe similar SIRPα lymph DC populations in sheep as those described in cattle and rat. Moreover, the molecular basis for the T stimulatory differences seen in cattle SIRPα DC populations are not fully understood (Howard et al., 2002). *Ex-vivo* sheep lymph SIRPα DC populations were characterised by surface phenotype at the protein level, and patterns of cytokine expression at the mRNA level. These studies aim to further or understanding of ovine DC populations and mammalian lymph DCs *in vivo*.

5.2 RESULTS

5.2.1 Animals and Experimental Procedures

Data presented in this chapter were derived from four sheep; Sheep 1 Sheep 2, Sheep 3 and Sheep 4 (Section 2.1)

Unless otherwise stated, the cells used in the experiments described in this chapter were freshly isolated afferent lymph cells (lymph cells). Cell phenotype was determined by immuno-labelling and flow cytometry. The monoclonal antibodies (mAb) used in the experiments described in this Chapter are illustrated in Table 5.1, and further details are given in Appendix 3. A description of immuno-labelling procedures is given in Section 2B.8. Briefly, cells were single, or double labelled with mouse mAb and biotin-conjugated mouse or rat mAb, or mouse / rat serum (negative controls). Specific mAb were detected with anti-mouse IgG conjugated to FITC, or biotinylated mAb with S:PE.

5.2.2 Flow Cytometry Analysis Parameters: Definition of Afferent Lymph Leucocytes.

Afferent lymph contains a number of leucocyte types including T cells, B cells, DCs (also termed afferent lymph veiled cells [ALVC]), and monocytes / macrophages (Hopkins et al., 1985). Lymph cell populations were identified with specific mAb: anti CD4 and CD8 (ST4 and SBU-T8, respectively (Maddox, Mackay and Brandon, 1985) for $CD4^+$ and $CD8^+$ lymphocytes; mAb clone IAH-CC15 recognising the WC1 antigen on γδ-T cells (Clevers et al., 1990); VPM30, which reacts with surface antigens found primarily on B cells, but also on activated T cells ($CD25^+$ $CD4^+$ / $CD8^+$) (Campbell et al., 1998); anti-CD11b recognising monocytes and macrophages, and the mAb OM1 specific for CD11c expressed on lymph DC and macrophages (Gupta et al., 1995). Human and murine DCs also express CD11c (O'Doherty et al., 1994; Vremec and Shortman, 1997), and this molecule is used as a DC marker in mice.

Table 5.1 Antibodies used in the experiments described in Section 5.2.

mAb Code	Specificity
IAH-CC6	CD2
ST4	CD4
SBU-T8	CD8
F10-150	CD11a
IL-A15	CD11b
OM1	CD11c
VPM67	CD14
VPM64	CD32
VPM63	CD16
IL-A156	CD40
73B	CD45RA
T11TS	CD58
IL-A159	CD80
IL-A190	CD86
3.29B1	CD206
SW73.2	MHC II
VPM54	MHC II
IAH-CC15	WC1
IL-A53	WC6
IL-A24	SIRPα
VPM30	B cells

Lymph cells were analysed for reactivity to the mAb mentioned above. Positive cells were gated and the light scatter properties determined, these are shown in Figure 5.2b. As expected, T lymphocytes ($CD8^+$, $CD4^+$, γδ T cells) displayed low FSC vs. SSC (FSC 200 - 400; SSC 100 - 200), as they are generally spherical, dense cells that are relatively small in comparison to other lymph cell populations. The mAb VPM30 identified cells with light scatter properties indicative of small, dense un-activated lymphocytes, and also cells with moderate FSC vs. SSC (FSC 400 – 500, SSC 200 – 400), which demonstrates they are larger, and possibly activated. $CD11c^+$ cells (DCs) displayed moderate-high FSC and SSC (FSC 400 – 1000; SSC 200 – 1000). $CD11b^+$ myeloid cells fell in both low and moderate-high FSC, SSC regions. Populations of cells expressing MHC II at intermediate (1 – 2-logs fluorescence) levels versus high levels (2 - 3-logs fluorescence) were analysed (Fig. 5.2b). A major proportion of lymph cells (53.7%) expressed intermediate levels of MHC II, and consisted primarily of small dense cells, but also with cells of moderate-high FSC SSC, which collectively may represent B cells and macrophages. The MHC II high population were large, complex cells (moderate - high FSC SSC), comprising a similar proportion of afferent lymph cells as did $CD11c^+$ cells (9.5% vs. 9%, respectively). Lymph cells expressing MHC II over 2-logs fluorescence displayed similar light scatter properties to the $CD11c^+$ population, and were thus likely to represent lymph DCs.

5.2.3. Characterisation of Ovine Lymph DC Markers.

Previous studies demonstrated that ovine afferent lymph DCs display moderate – high FSC SSC light scatter properties (Bujdoso et al., 1989), and express high levels of MHC II, above that on B cells and macrophages (Hopkins et al., 1985). The experiments described in Section 5.2.2. demonstrated that $CD11c^+$ and MHC II^{hi} cells display morphological characteristics (light scatter properties) indicative of DCs. CD11c expression is modulated on murine and human DCs during maturation. Mature DCs in lymphoid tissues express high levels of CD11c, whereas immature DCs express CD11c with heterogeneity (Steinman, Pack and Inaba, 1997). Similarly, surface MHC II expression is markedly up-regulated during DC maturation (Cella et al., 1997).

Figure 5.2 Phenotype and flow cytometric light scatter properties of afferent lymph cell populations. Afferent lymph cells were stained for MHC II using biotin-conjugated SW73.2 (Table 5.1), that was labelled with streptavidin-PE and detected on the FL2 channel. Cells were double-labelled with a second, mouse mAb and stained with anti-mouse IgG:FITC conjugate, and detected on the FL1 channel. Negative controls samples were incubated with normal mouse serum (1:500) or biotinylated normal mouse serum (1:100) and secondary FITC and PE conjugates, respectively. Details of the flow cytometers, cytometer settings, and analysis parameters are given in Section 2B.8.4. 10 000 events were analysed in each sample. Samples were performed in triplicate. The data is representative of two independent experiments performed on cells derived from Sheep 1.

a) Scatter profile of lymph cell populations. No gate was applied.

b) The antigen being analysed is shown with each histogram, staining is shown in the coloured profile. Negative control staining is shown as the open profile. Light scatter profiles of positive cells are presented. Gates used for analysis are indicated on the histograms (R1), and percentage of positive gated cells are shown with each histogram.

Figure 5.2a

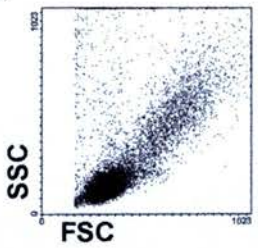


Figure 5.2b

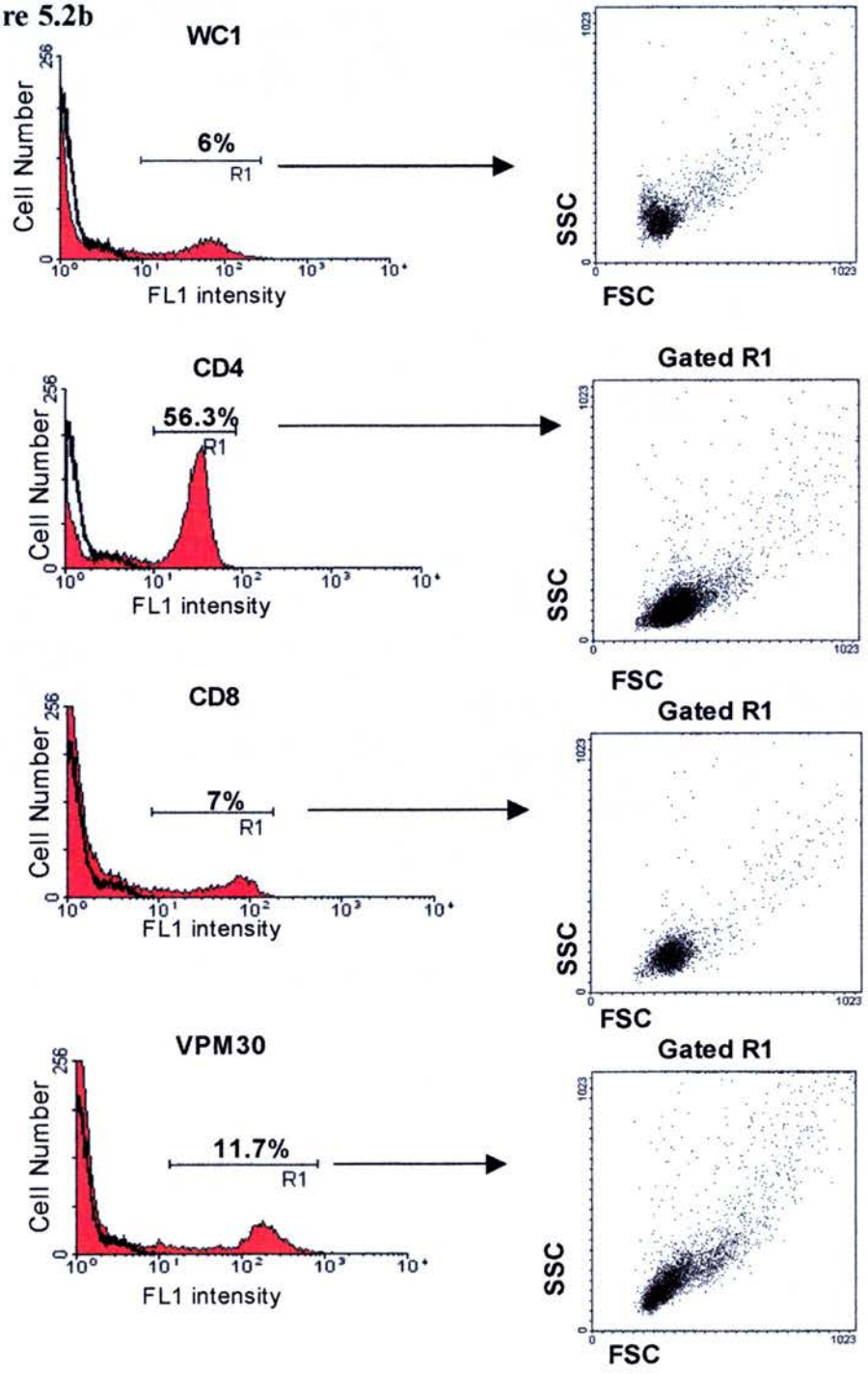
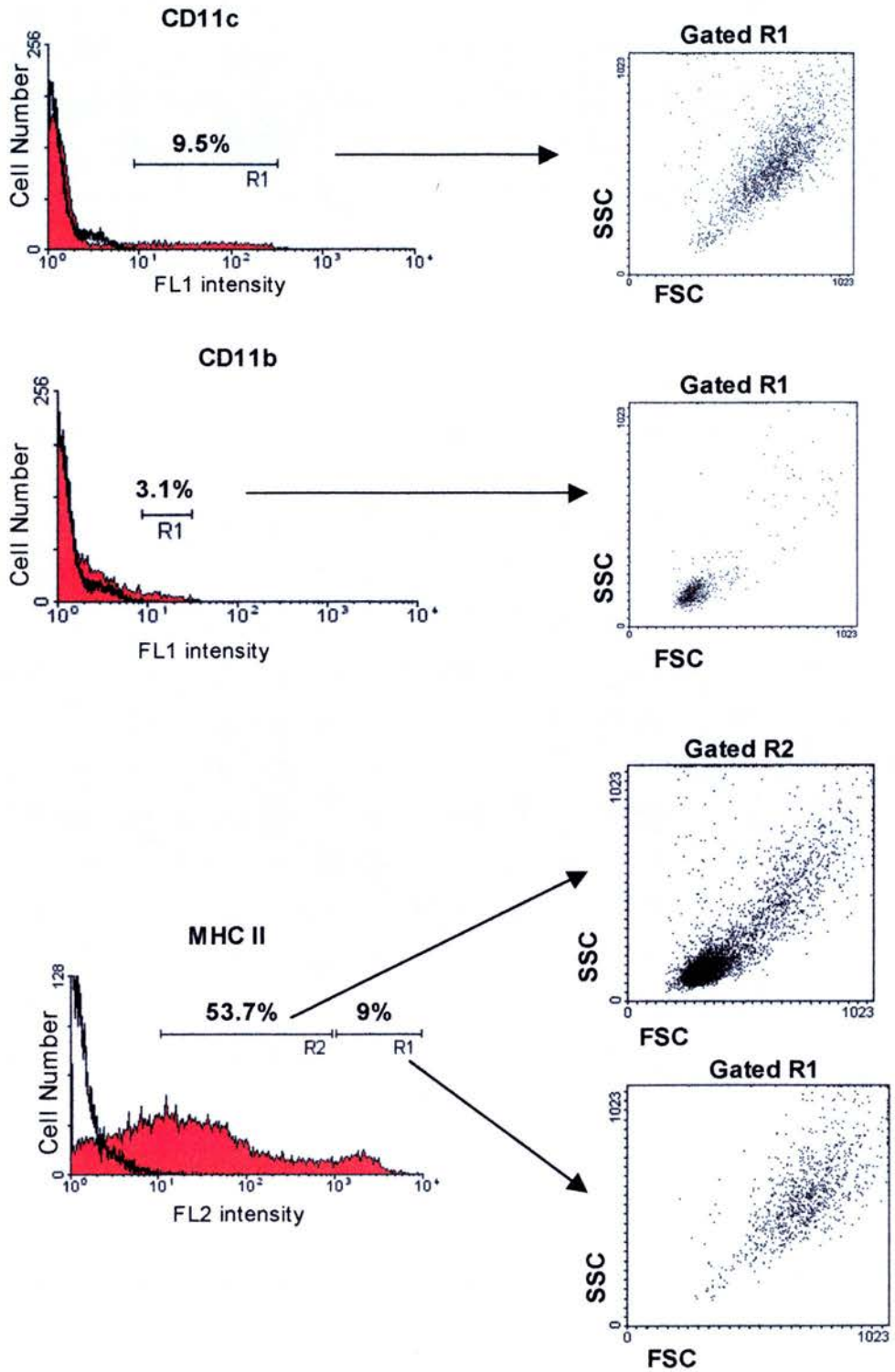


Figure 5.2b


CD11c
Cell Number
9.5%
R1
FL1 intensity
Gated R1
SSC
FSC
CD11b
3.1%
R1
FL1 intensity
Gated R1
SSC
FSC
MHC II
53.7%
9%
R2
R1
Cell Number
FL2 intensity
Gated R2
SSC
FSC
Gated R1
SSC
FSC

DCs undergo maturation during migration to secondary lymphoid tissues. It was helpful, therefore, to identify a molecule that could be utilised for identifying lymph DCs at different stages of maturation.

Ovine lymph DCs and a small proportion of lymphocytes express the WC6 antigen (Dutia, Ross and Hopkins, 1993). To investigate whether lymph DCs could be defined by light scatter properties and WC6 expression, lymph cells were stained with anti-MHC II:biotin and anti-WC6 mAb. As shown in Figure 5.3, 89.6% of MHC II^{hi} DCs expressed WC6. Additionally, cells co-expressing WC6 and MHC II at 2 - 3-logs fluorescence displayed light scatter properties indicative of DCs (Section 5.2.2.). Based on these results, WC6 expression and moderate – high FSC were useful characteristics for defining afferent lymph DCs.

5.2.4 Surface Phenotype of Lymph DCs

Surface marker expression of lymph DCs was characterised using the panel of mAb listed in Table 5.1. Freshly isolated afferent lymph cells were gated for DCs based on moderate – high FSC and WC6 expression at 1 – 2 logs fluorescence (Fig. 5.4). Percentage positive cells over that of negative controls (cells stained with mouse serum and secondary conjugates) determined the degree of marker expression. DC phenotype was analysed in three sheep with two independent experiments performed on cells from Sheep 1, three experiments on Sheep 2, and a single experiment on Sheep 3. Pooled data are presented in Figure 5.5 and are referred to in the text below. Surface phenotype of DCs from Sheep 2 represented two out of three animals tested (Section 5.2.5), therefore, the flow cytometric histograms derived from Sheep 2 are presented in this section describing lymph DC phenotype. Representative antigen expression profiles on DCs from Sheep 2 are shown in Figure 5.4.

DCs expressed the SIRPα molecule with marked heterogeneity (Fig. 5.4). SIRPα expression clearly split the cells into positive and negative fractions, 51.4% (± 4.3%) DCs expressed SIRPα as a single population, whereas the remainder (48.6% ± 4.3%) were negative for this antigen. DC expression of CD11c was similar to the experiments performed in Section 5.2.2, a large proportion were $CD11c^{+}$ (83.5% [± 1.8%]) with levels detected at 1 – 2.5-logs fluorescence.

Figure 5.3. Light scatter characteristics of WC6$^+$ afferent lymph cells. Freshly isolated afferent lymph cells were analysed by flow cytometry following staining mouse anti ruminant WC6 and anti-MuIgG:FITC (FL1 channel) and anti ruminant MHC II (SW73.2: biotin) and streptavidin:PE (FL2 channel). **a)** Forward (FSC) and side (SSC) angle scatter of afferent lymph cells. 10 000 events were analysed in each sample. **b)** MHC II and WC6 expression on afferent lymph cells. No gating was applied. Double positive cells displaying high levels (> 3 logs fluorescence) of MHC II expression were gated for analysis as indicated. FSC and SSC characteristics of MHC II^{hi} WC6$^+$ cells are shown. **c)** Cells with mod - high FSC and MHC II levels at 3 logs fluorescence were gated and analysed for WC6 expression. Negative control staining is shown with the black histogram outline, staining with the red histogram outline. Percentage positive cells are shown with each profile. Cells were derived from sheep 2. The data shown is representative of two experiments with triplicate samples.

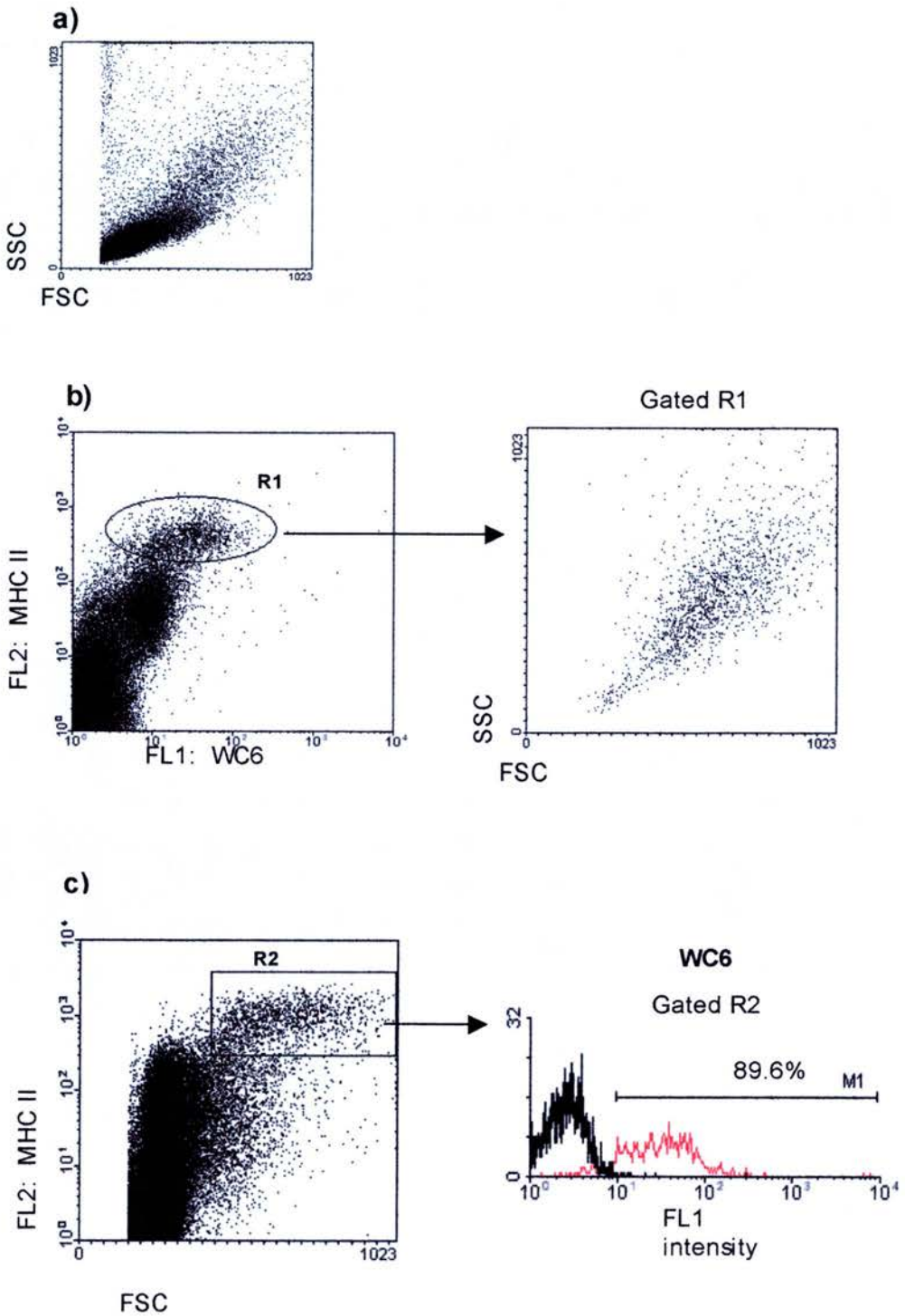
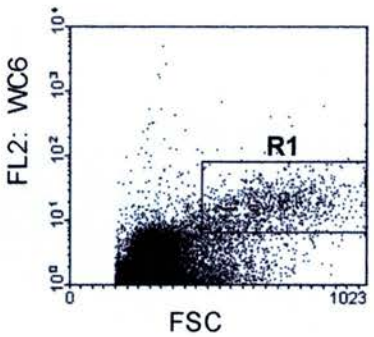
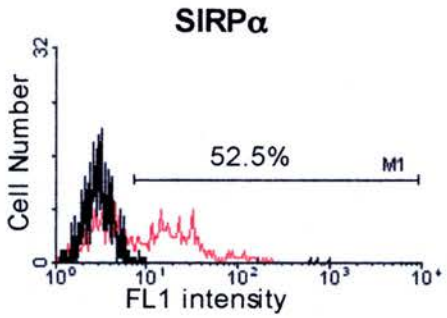
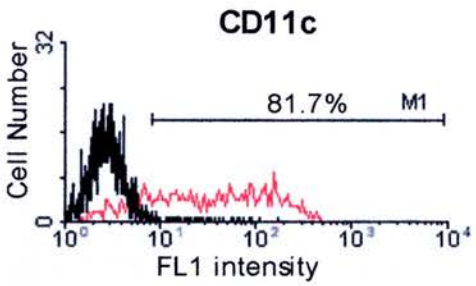
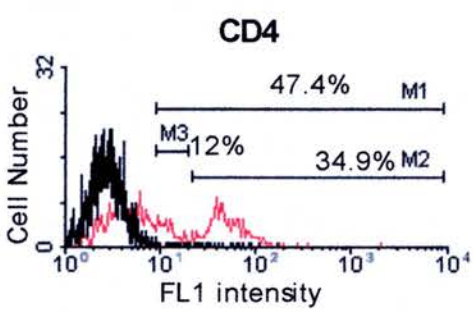

a)
SSC
FSC
1023
0
b)
R1
FL2: MHC II
FL1: WC6
Gated R1
SSC
FSC
c)
R2
FL2: MHC II
FSC
WC6
Gated R2
89.6%
M1
FL1
intensity

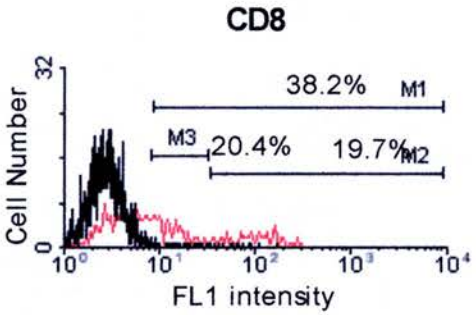
Figure 5.4 Lymph dendritic cell surface phenotype. Afferent lymph cells were stained with anti-WC6:biotin conjugate, labelled with streptavidin-PE and detected on the FL2 channel. Cells were double-labelled with a second, mouse mAb and stained with anti-mouse IgG:FITC conjugate, and detected on the FL1 channel. Negative controls samples were incubated with normal mouse serum (1:500) or biotinylated normal mouse serum (1:100) and secondary FITC and PE conjugates, respectively. Details of the flow cytometers, cytometer settings, and analysis parameters are given in Section 2B.8.3. Dendritic cells (DC) were gated as the $WC6^+$ cell population with moderate – high FSC, as shown (R1). 10 000 events were analysed in each sample, samples were performed in duplicate. The antigen being analysed is shown with each histogram, staining is shown as the red profile. Negative control staining is shown as the black profile. Percentage of positive gated cells are shown with each histogram. The data is representative of three independent experiments performed on cells derived from Sheep 2.

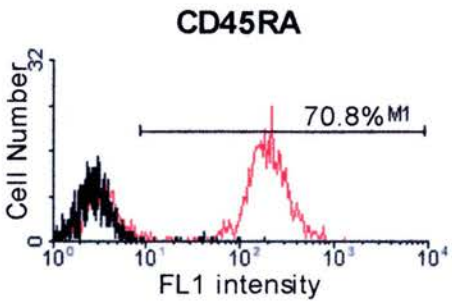

FL2: WC6
R1
FSC
0
1023

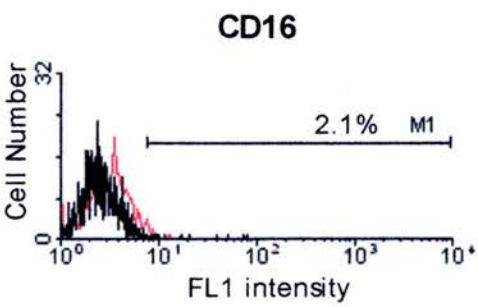

SIRPα
Cell Number
52.5%
M1
FL1 intensity

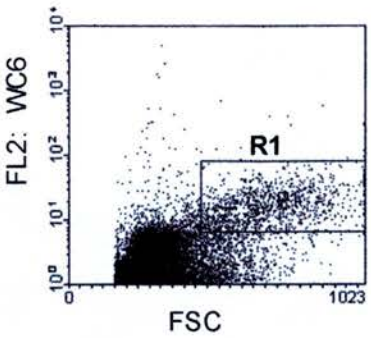

CD11c
Cell Number
81.7%
M1
FL1 intensity

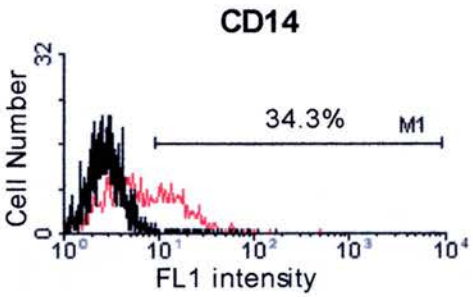

CD4
Cell Number
47.4%
M1
M3
12%
34.9%
M2
FL1 intensity

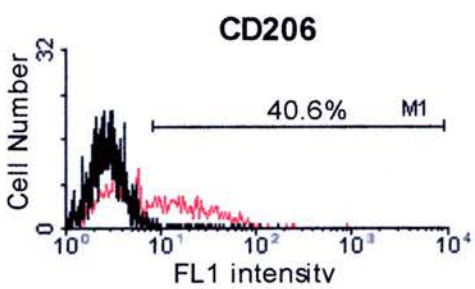

CD8
Cell Number
38.2%
M1
M3
20.4%
19.7%
M2
FL1 intensity

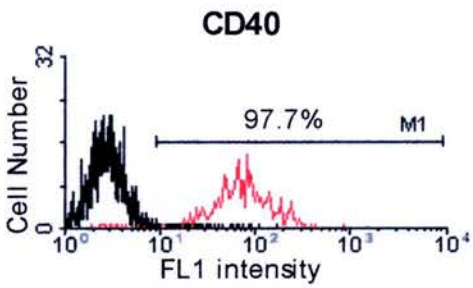

CD45RA
Cell Number
70.8%
M1
FL1 intensity

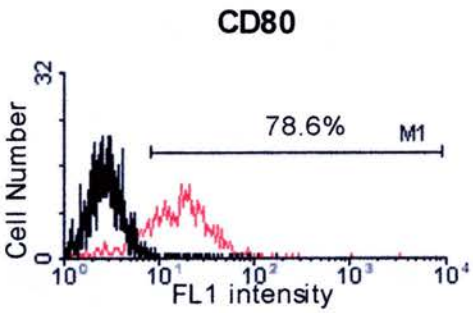

CD16
Cell Number
2.1%
M1
FL1 intensity

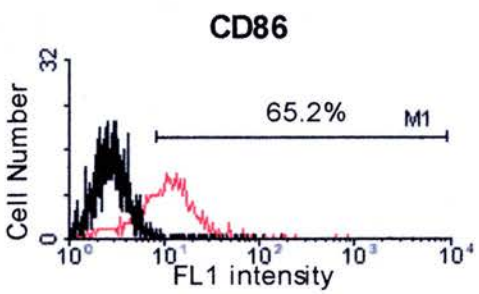

FL2: WC6
R1
FSC
0
1023

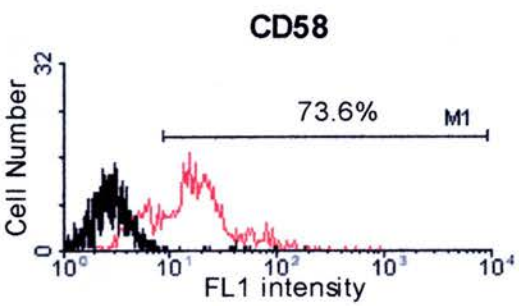

CD14
34.3%
M1
Cell Number
FL1 intensity


CD206
40.6%
M1
Cell Number
FL1 intensitv


CD40
97.7%
M1
Cell Number
FL1 intensity


CD80
78.6%
M1
Cell Number
FL1 intensity


CD86
65.2%
M1
Cell Number
FL1 intensity


CD58
73.6%
M1
Cell Number
FL1 intensity

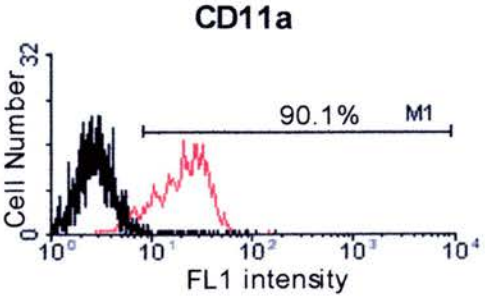
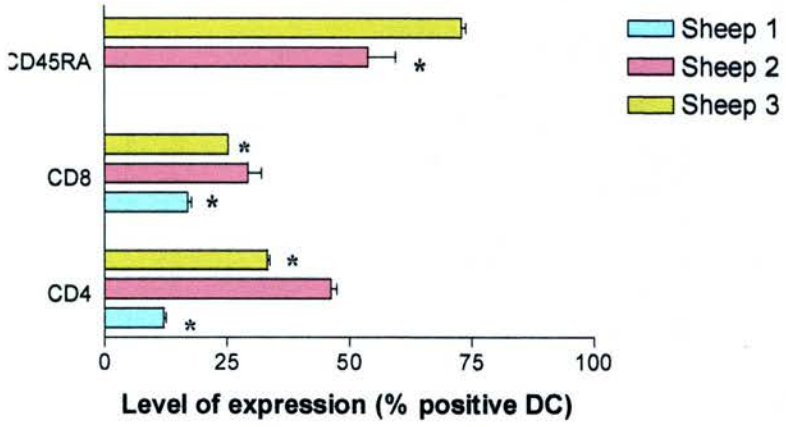
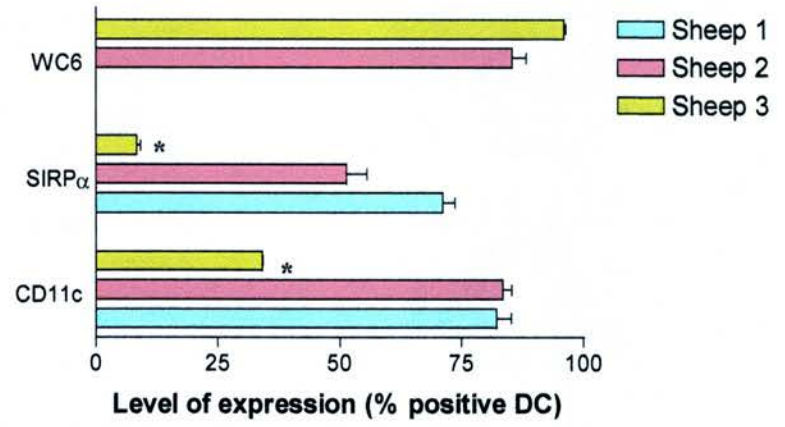
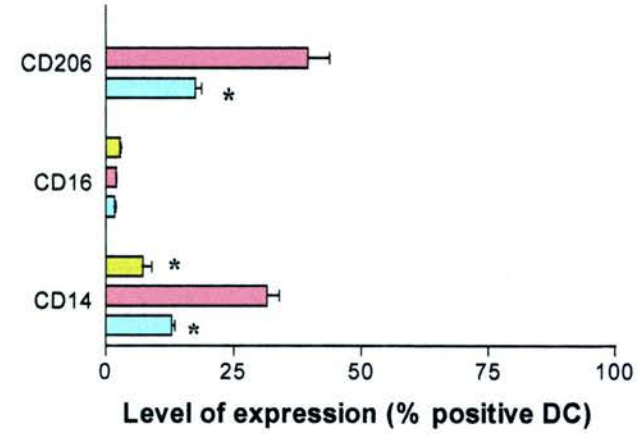
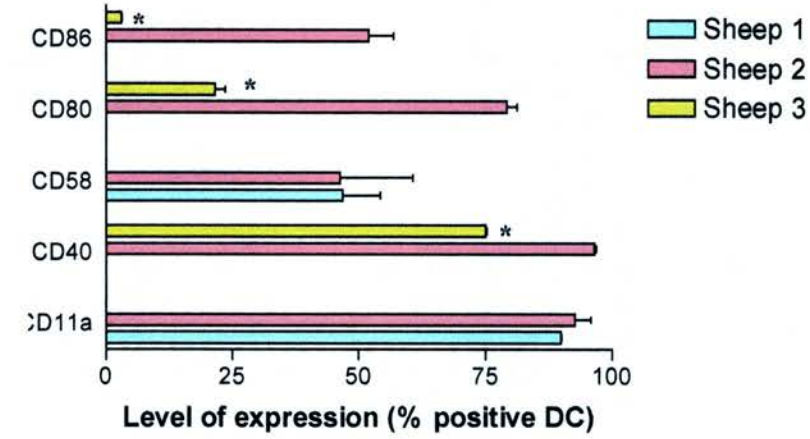

CD11a
90.1%
M1
Cell Number
FL1 intensity

Figure 5.5 DC phenotype: A comparison of lymph DC from three sheep. Surface phenotype of freshly isolated lymph DCs from three sheep (Sheep 1, Sheep 2 and Sheep 3) was analysed by flow cytometry. The level of antigen expression was determined by the extent of staining (percentage [%] positive DCs) over cells stained with normal mouse serum (1:500) and secondary conjugates as controls. Data represents the mean and error bars standard error of the mean, level of expression from two experiments for Sheep 1, three experiments for Sheep 2, and one experiment for Sheep 3 with duplicate samples. An asterix (*) denotes a significant difference ($p < 0.05$; using a two-tailed t test) in the mean level of expression for an antigen. The mAb against CD40, CD80 and CD86 were not available during analysis of cells from Sheep 1, so were not included in this analysis.


CD45RA
CD8
CD4
Sheep 1
Sheep 2
Sheep 3
0
25
50
75
100
Level of expression (% positive DC)


WC6
SIRPα
CD11c
Sheep 1
Sheep 2
Sheep 3
0
25
50
75
100
Level of expression (% positive DC)


CD206
CD16
CD14
Sheep 1
Sheep 2
Sheep 3
0
25
50
75
100
Level of expression (% positive DC)


CD86
CD80
CD58
CD40
CD11a
Sheep 1
Sheep 2
Sheep 3
0
25
50
75
100
Level of expression (% positive DC)

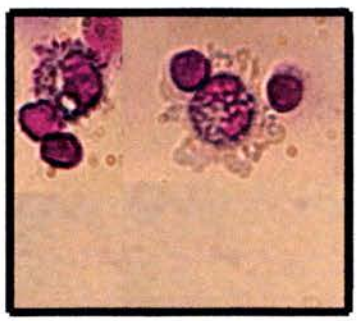
DCs displayed low and intermediate expression of CD4 and CD8, low levels were evident on 11.8% (± 0.3%) and 16.4% (± 3%) DCs, respectively (populations indicated in Fig 5.4). The remainder of DCs positive for both CD4 (36.6% [± 0.8%]) and CD8 (18.7% [2.2%]) displayed levels over 2-logs fluorescence that appeared to be single populations of cells (Fig. 5.4). This was surprising, as DCs have been reported to express low levels of CD4 and CD8 in mouse and man (Olweus et al., 1997; Pulendran et al., 1997; Anjuere et al., 1999; Cella et al., 1999; Trinite et al., 2000) however, expression of these antigens at 2-logs fluorescence were indicative of lymphocytes (Figure 5.2b). Afferent lymph DCs have been documented to consistently form antigen independent clusters with lymphocytes (Pugh, MacPherson and Steer, 1983; Hein, McClure and Miyasaka, 1987; Cumberbatch, Illingworth and Kimber, 1991; Brand et al., 1999). Cytological preparations of afferent lymph cells were analysed by microscopy following sequential double labelling with mAb as performed for flow cytometric analysis. Cells of typical DC morphology were evident clustered with cells of lymphocyte characteristics (Figure 5.6a). Furthermore, $WC6^+$ $CD4^+$ and $WC6^+$ $CD8^+$ cells have light scatter properties characteristic of DCs (ie. moderate – high FSC, SSC) (Figure 5.6b), which are not typical of afferent lymph lymphocytes that display low FSC, SSC (Fig. 5.2). Together, this evidence suggests the cells in the DC gate displaying CD4 and CD8 expression at 2-logs fluorescence probably represent DC:lymphocyte clusters.

Expression of CD45RA by lymph DCs showed marked heterogeneity (Fig. 5.4). Distinct positive and negative populations were demonstrated, 53.7% (± 5.8%) of DCs expressed CD45RA over 2-logs fluorescence.

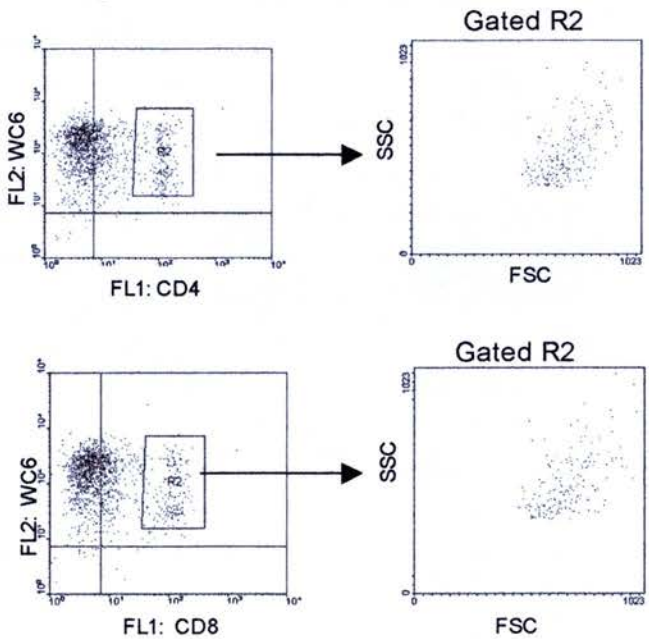
Of molecules involved in antigen detection and sampling, lymph DCs were basically negative for CD16 (2% [± 1%] positive staining), whereas considerable proportions of DCs clearly expressed CD14 (31.6% [± 2.5%]) and CD206 (39.6 [± 4.2%]) (Fig. 5.4).

Most of the DCs expressed the co-stimulatory molecules analysed, population shifts on FL1 were evident for CD40, CD80, CD86, and CD58 staining (Figure 5.4). The highest expressed of these molecules was CD40, 96.5% (± 3.2%) DCs displayed homogenous expression of CD40 at 2-logs fluorescence. DCs also expressed

Figure 5.6. Demonstration of DC:Lymphocyte clusters. Following sequential double immuno-labelling, afferent lymph cells were analysed by cytocentrifugation and Giemsa staining (a) and flow cytometry (b).



a) Cells of typical DC morphology; irregular plasma membrane with short processes and / or pseudopodia are evident, clustered with smaller, spherical cells of lymphocyte morphology. Original magnification x 1,250.



b) Afferent lymph cells were stained with mAb against WC6 and CD4, or CD8 and secondary conjugates. DCs with moderate-high FSC and SSC were gated for analysis. Negative controls cells were incubated with serum and secondary conjugates, and quadrants set so 99% of the negative control staining fell within the bottom left quadrant. Discrete populations of double positive cells were gated (R2), FSC SSC characteristics are shown in the dot plots.

CD80 and CD86 as a single population, although expression levels differed in that 79.2% (± 2%) displayed positive staining for CD80, whereas 51.9% (± 4.9%) DCs displayed CD86 staining above negative controls. Levels of CD58 were similar with that of CD86 (1 – 2 logs fluorescence), although CD58 showed some heterogeneity of expression (Fig. 5.4). Most of the DCs also expressed the adhesin CD11a, expression was homogenous with expression on 92.7% (± 3.2%) DCs (Fig. 5.4).

5.2.5. DC Heterogeneity in Sheep

DC phenotype was compared between Sheep 1 and Sheep 2, and significant differences in expression levels were demonstrated for CD4 (mean difference 30%, p <0.001), CD14 (mean difference 18%, p <0.01), CD206 (mean difference 22%, p <0.05), and SIRPα (mean difference 20%, p <0.01). DC expression of MHC II, CD8, CD16, CD11a, CD58 and CD11c were similar between Sheep 1 and 2 (Fig. 5.5).

DCs from Sheep 3 were analysed and compared with DCs from Sheep 1 & 2. Other than CD45RA and WC6, levels of all other surface markers analysed were significantly lower in Sheep 3 compared with both Sheep 1 and Sheep 2. The profile of MHC II expression was markedly different for Sheep 3 compared with Sheep 1 and 2; 39.3% DCs from Sheep 3 expressed MHC II at 3-logs fluorescence whereas over 80% of DCs from Sheep1 and Sheep 2 expressed high levels of MHC II (Fig. 5.6). Similarly, 1% of afferent lymph cells expressed MHC II over 3-logs fluorescence in Sheep 3, whereas 10% of these cells in Sheep 1 expressed MHC II at high levels (Fig. 5.7). The proportion of $WC6^{+}$: FSC moderate cells in Sheep 3 were similar with Sheep 2 (85.4% [± 2.9%] vs. 96% [± 0.3%], respectively). However, that a minor fraction (39.3%) of Sheep 3 DCs were MHC II^{hi} indicated that the DCs from Sheep 3 were at an earlier stage of maturation, or, that the WC6:FSC moderate population contained cells other than DCs, such as activated lymphocytes. Different proportions of lymph leucocyte populations in Sheep 3 could reflect individual physiological differences, or indicate the presence of infection. However, the presences of infection (oedema, increase in polymorphonuclear cells [specifically neutrophils] which would have high SSC, low FSC) were not evident.

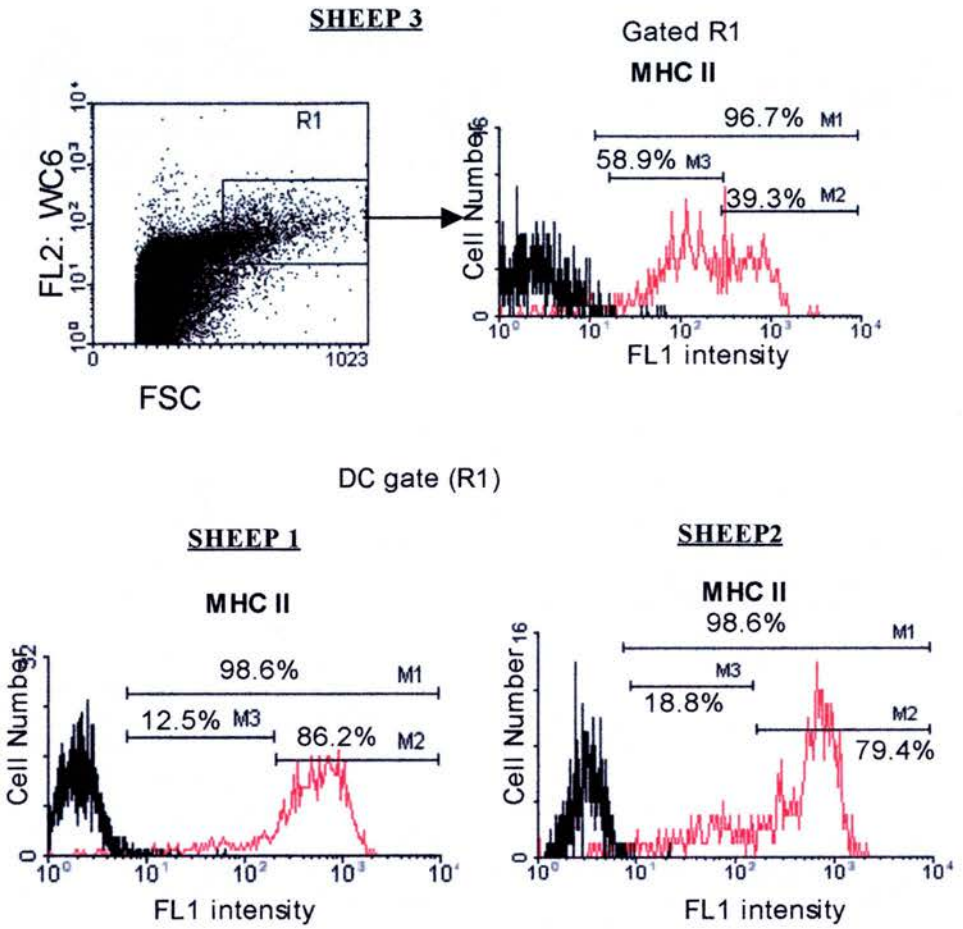


Figure 5.7 MHC II expression on DC from individual sheep. Afferent lymph DC were gated according to WC6 expression and moderate – high FSC, as indicated in R1. Using identical gating criteria on lymph cells derived from Sheep 1, Sheep 2 and Sheep 3, MHC II expression was analysed on the FL1 channel. Staining with mouse serum (1:500) and secondary conjugates is shown as the black histogram. MHC II-positive cells were gated (M1). Levels of MHC II between 1 – 2 logs fluorescence (moderate [M3]) and over 2 logs fluorescence (high [M2]) were gated. The percentage positive cells are shown with each gate.

5.2.6 SIRPα DC Populations

Afferent lymph cells from Sheep 1 and Sheep 2 were used to determine the surface phenotype of SIRPα$^+$ and SIRPα$^-$ DCs. DCs were gated with moderate to high FSC and SSC (Section 5.2.2.), and analysed by two-colour flow cytometry similar to previous experiments. Two independent experiments were performed on cells from Sheep 1 and Sheep 2, and the data from duplicate samples pooled (n = 8). Marker expression of SIRPα$^+$ and SIRPα$^-$ DCs is presented in Table 5.2, these data are referred to in the text. Flow cytometric dot plots and histograms from a representative experiment are shown in Figure 5.8.

The DC populations differed significantly in CD11c expression (p = <0.05). More or less all SIRPα$^+$ DCs were CD11c$^+$ (99.8% [± 0.3%]), whereas 53% (± 4.1%) SIRPα$^-$ DCs did not express CD11c, the CD11c$^+$ fraction of SIRPα$^-$ DCs displayed lower expression compared with SIRPα$^+$ (Fig 5.7). Similarly, the majority of SIRPα$^+$ DCs expressed WC6 (92% [± 0.1%]), where as this antigen was found on a significantly lower percentage of SIRPα$^-$ DCs (77.3% [± 4.7%]; p = <0.05).

While the proportions of CD4 staining on SIRPα$^+$ and SIRPα$^-$ DC did not differ significantly (p = 0.4; Table 5.2), patterns of CD4 expression showed heterogeneity between the DC populations. As shown in Figure 5.7, the SIRPα$^-$ DC fraction contained a higher proportion of CD4$^-$ (no shift on FL1) cells compared with SIRPα$^+$ DC (75.1% [± 3.9%] vs. 63.4% [± 11.6%], respectively), and a distinct population of cells displaying CD4 staining at 1.5-logs fluorescence were more evident in the SIRPα$^+$ DC fraction. Both DC populations appeared to contain similar levels of cells expressing low levels of CD4 (Fig. 5.7). CD8 reactivity in the DC populations was similar (SIRPα$^+$; 32.1% [± 10.4%] positive DC, and SIRPα$^-$; 19.6% [± 5.6%] positive DC; p = 0.8), as was patterns of CD8 expression (Fig. 5.7). SIRPα$^+$ and SIRPα$^-$ DC both contained CD8$^-$ cells, cells expressing low levels of CD8 and fractions of CD8 expression 2-logs fluorescence (Fig. 5.7).

One of the most striking differences in marker expression between the SIRPα DC populations was CD45RA expression (Fig. 5.7). SIRPα$^+$ DC expression of CD45RA was negligible (7% ± 0.5%) and from the dot plots seemed to represent

Table 5.2 Surface marker expression on SIRPα$^+$ / SIRPα$^-$ lymph dendritic cells. Freshly isolated afferent lymph DCs were analysed following double immunolabelling, and flow cytometry, as described in the text. Percentage of positive cell staining above controls was determined for each marker, and data pooled (n = 8). Data were derived from four experiments performed on cells from Sheep 1 and Sheep 2. Mean percentage expressions, ± standard error of the mean, and the *p* value determined from unpaired two-tailed t tests are presented. *p* values less than 0.05 were considered significant.

Antigen	% SIRPα$^+$ DC	% SIRPα$^-$ DC	*p* value
WC6	92 (± 0.1)	77.35 (± 4.7)	0.020
CD11c	99.5 (± 0.3)	47 (± 4.1)	0.006
CD4	36.6 (± 11.6)	24.9 (± 3.9)	0.401
CD8	32.1 (± 10.4)	19.6 (± 5.6)	0.796
CD45RA	7 (± 0.5)	61.6 (± 2.8)	<0.001
CD16	4.5 (± 1)	1 (± 0.5)	0.022
CD14	41.62 (± 10)	3.6 (± 1)	0.011
CD206	56.9 (± 12.4)	6.2 (± 1.4)	0.017
MHC II	100	100	-
CD40	99.3 (± 1.7)	77.5 (± 2.2)	<0.001
CD80	72.5 (± 9.3)	31.7 (± 1.7)	0.039
CD86	28.9 (± 8.5)	13.8 (± 4.1)	0.163
CD58	87 (± 0.9)	53 (± 12.5)	0.054
CD11a	93 (± 1.7)	96.4 (± 0.5)	0.09

Figure 5.8 Phenotype of SIRPα DC populations. Afferent lymph cells were stained with anti-SIRPα:biotin conjugate, labelled with streptavidin-PE and detected on the FL2 channel. Cells were double-labelled with a second, mouse mAb and stained with anti-mouse IgG:FITC conjugate, and detected on the FL1 channel. Negative controls samples were incubated with normal mouse serum (1:500) or biotinylated normal mouse serum (1:100) and secondary FITC and PE conjugates, respectively. Details of the flow cytometers, cytometer settings, and analysis parameters are given in Section 2B.8.3. DCs were gated as the moderate - high FSC SSC population (FSC 400 – 1000; SSC 200 – 1000), as shown (R1). Density plots showing the degree of antigen expression on the SIRPα$^+$ / SIRPα$^-$ DCs are shown, percentage of gated cells are shown with each quadrant. The DC populations were gated: R2, SIRPα$^+$; R3, SIRPα$^-$ and extent of FL1 staining presented in a histogram. The antigen being analysed is shown with each histogram, staining is shown as the coloured profile; SIRPα$^+$ DCs purple, SIRPα$^-$ DCs green. Negative control staining is shown as the black profile. Percentage of positive gated cells are shown with each histogram. 10 000 events were analysed in each sample, samples were performed in duplicate. The data is representative of three independent experiments performed on cells derived from Sheep 2.

Figure 5.8

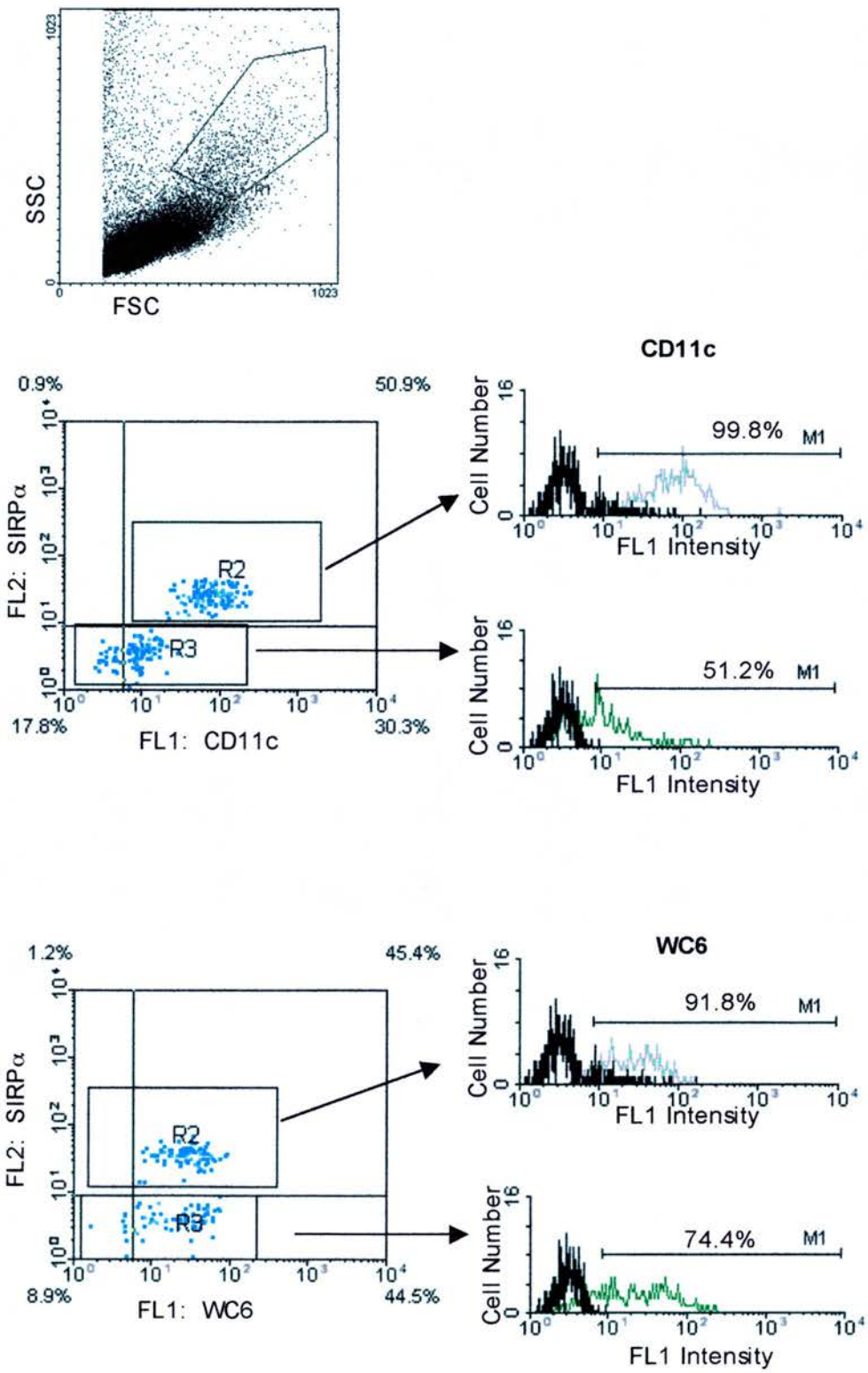

SSC
FSC
R1
CD11c
0.9%
50.9%
17.8%
30.3%
FL2: SIRPα
FL1: CD11c
R2
R3
Cell Number
99.8%
M1
FL1 Intensity
51.2%
WC6
1.2%
45.4%
8.9%
44.5%
FL1: WC6
91.8%
74.4%

Figure 5.8

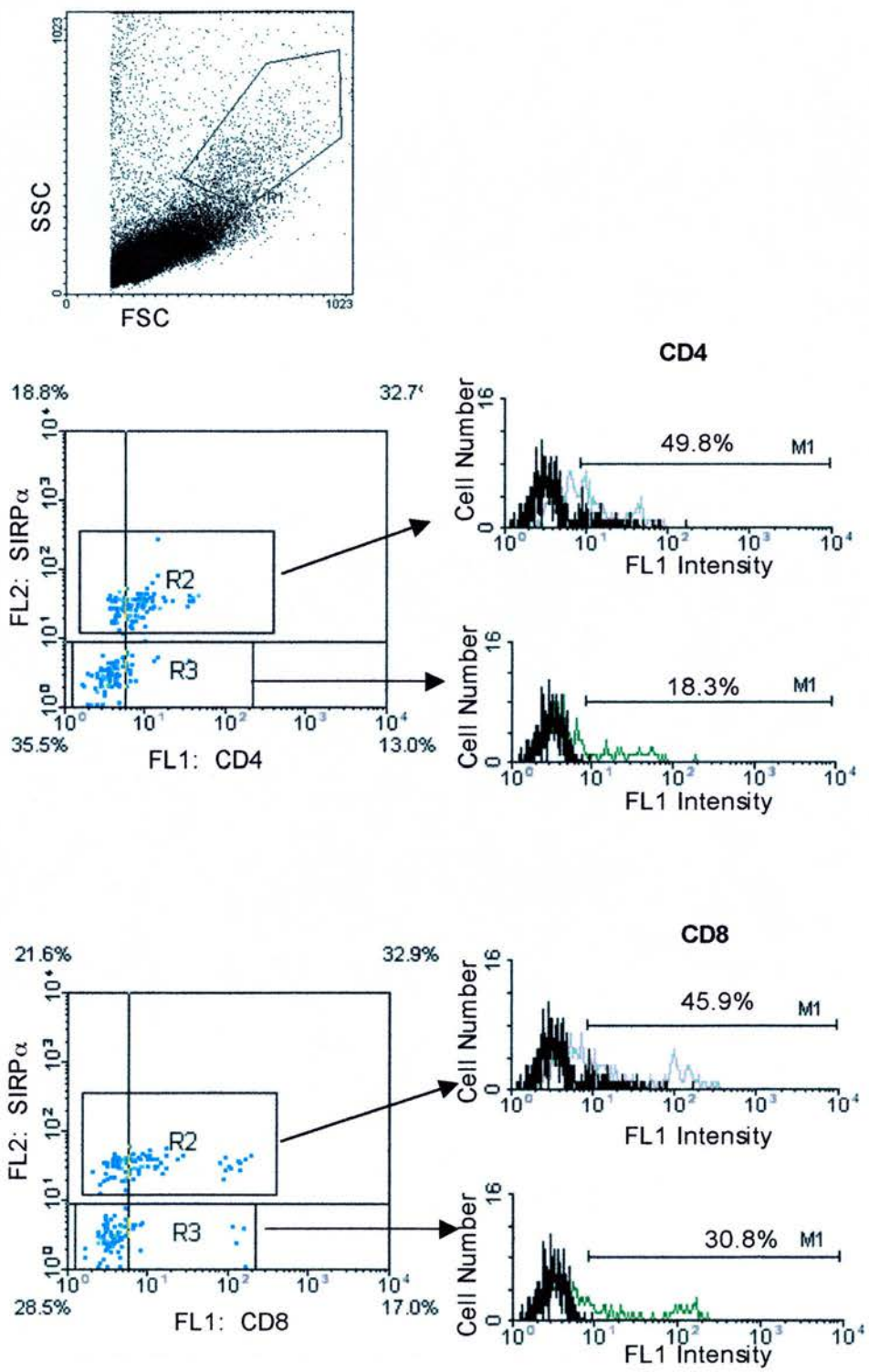

SSC
FSC
R1
CD4
18.8%
32.7%
FL2: SIRPα
FL1: CD4
R2
R3
35.5%
13.0%
Cell Number
49.8%
M1
FL1 Intensity
18.3%
CD8
21.6%
32.9%
FL1: CD8
28.5%
17.0%
45.9%
30.8%

Figure 5.8

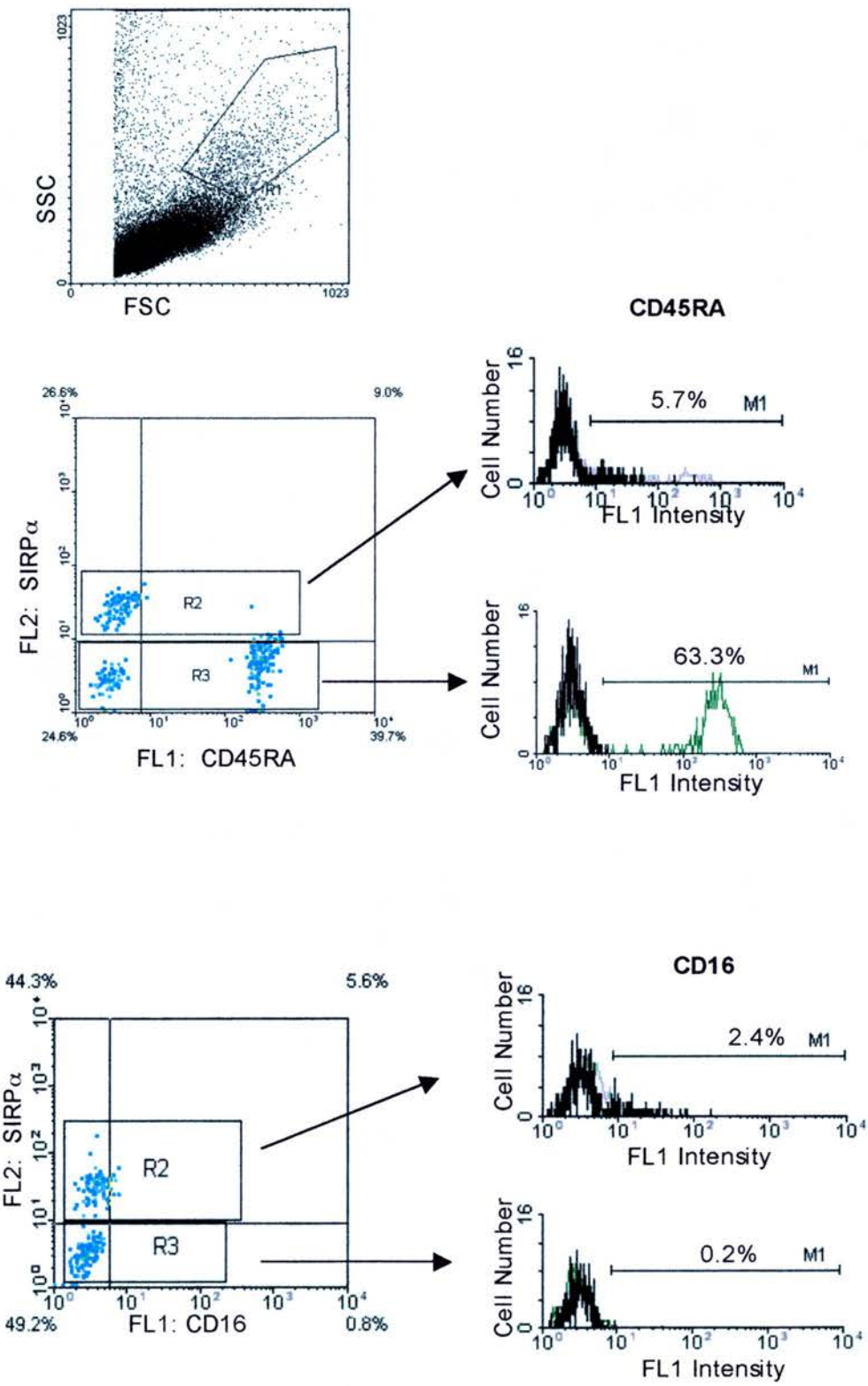

SSC
FSC
R1
CD45RA
FL2: SIRPα
FL1: CD45RA
R2
R3
26.6%
9.0%
24.6%
39.7%
Cell Number
FL1 Intensity
5.7%
M1
63.3%
CD16
44.3%
5.6%
49.2%
0.8%
2.4%
0.2%

Figure 5.8

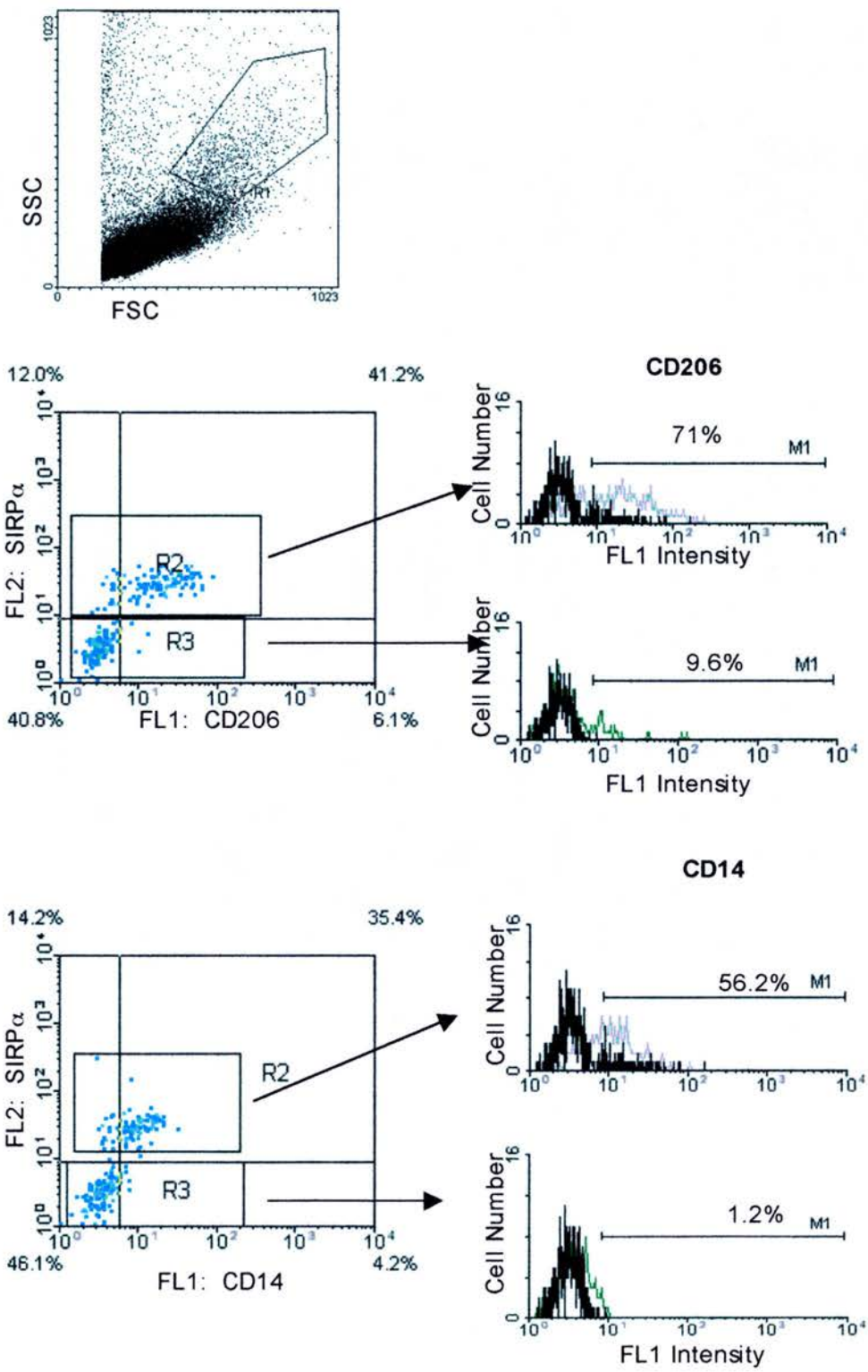

SSC
FSC
R1
12.0%
41.2%
FL2: SIRPα
R2
R3
40.8%
FL1: CD206
6.1%
CD206
Cell Number
71%
M1
FL1 Intensity
9.6%
14.2%
35.4%
46.1%
FL1: CD14
4.2%
CD14
56.2%
1.2%

Figure 5.8

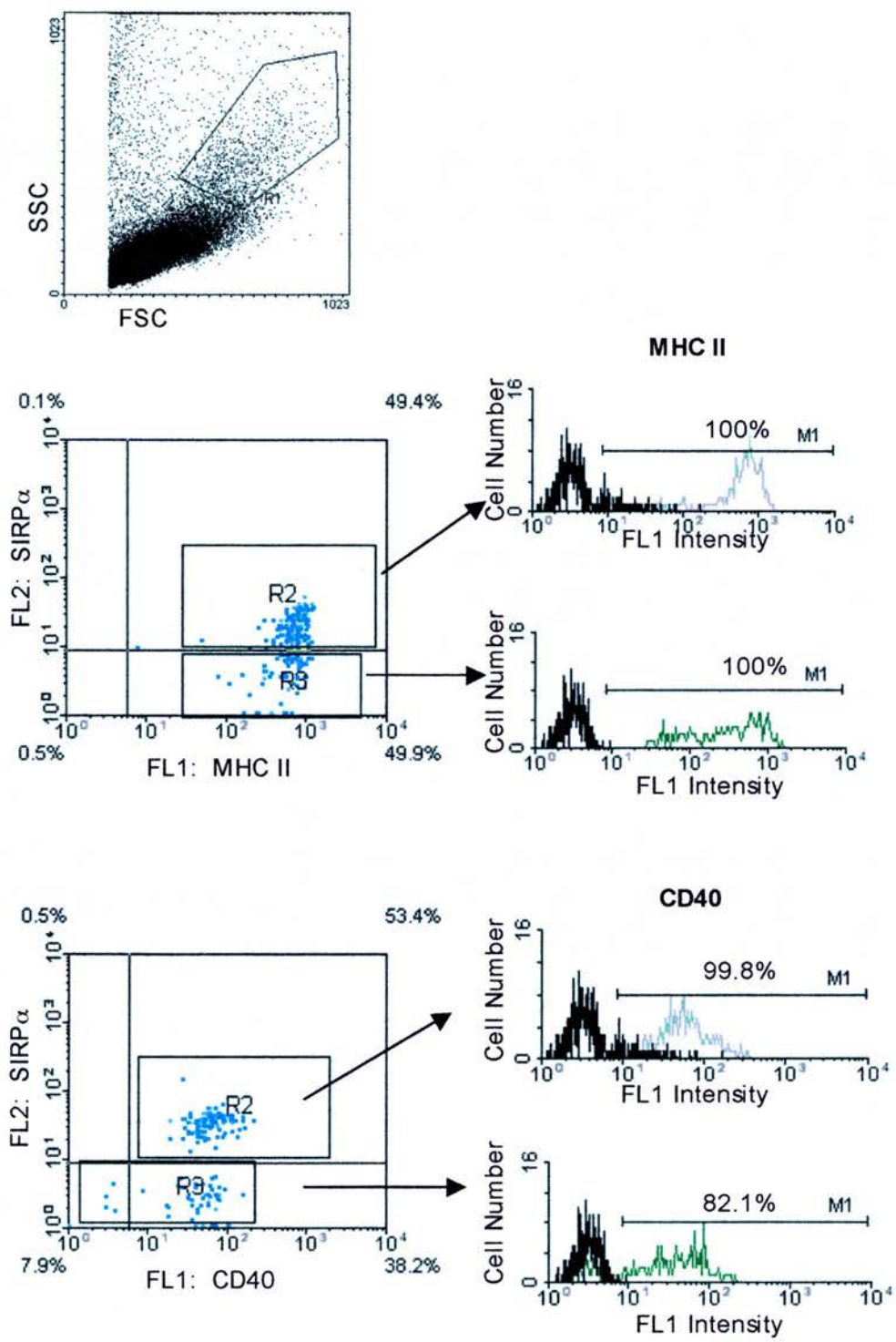

SSC
FSC
R1
MHC II
0.1%
49.4%
FL2: SIRPα
R2
R3
0.5%
49.9%
FL1: MHC II
Cell Number
100%
M1
FL1 Intensity
CD40
0.5%
53.4%
7.9%
38.2%
FL1: CD40
99.8%
82.1%

Figure 5.8

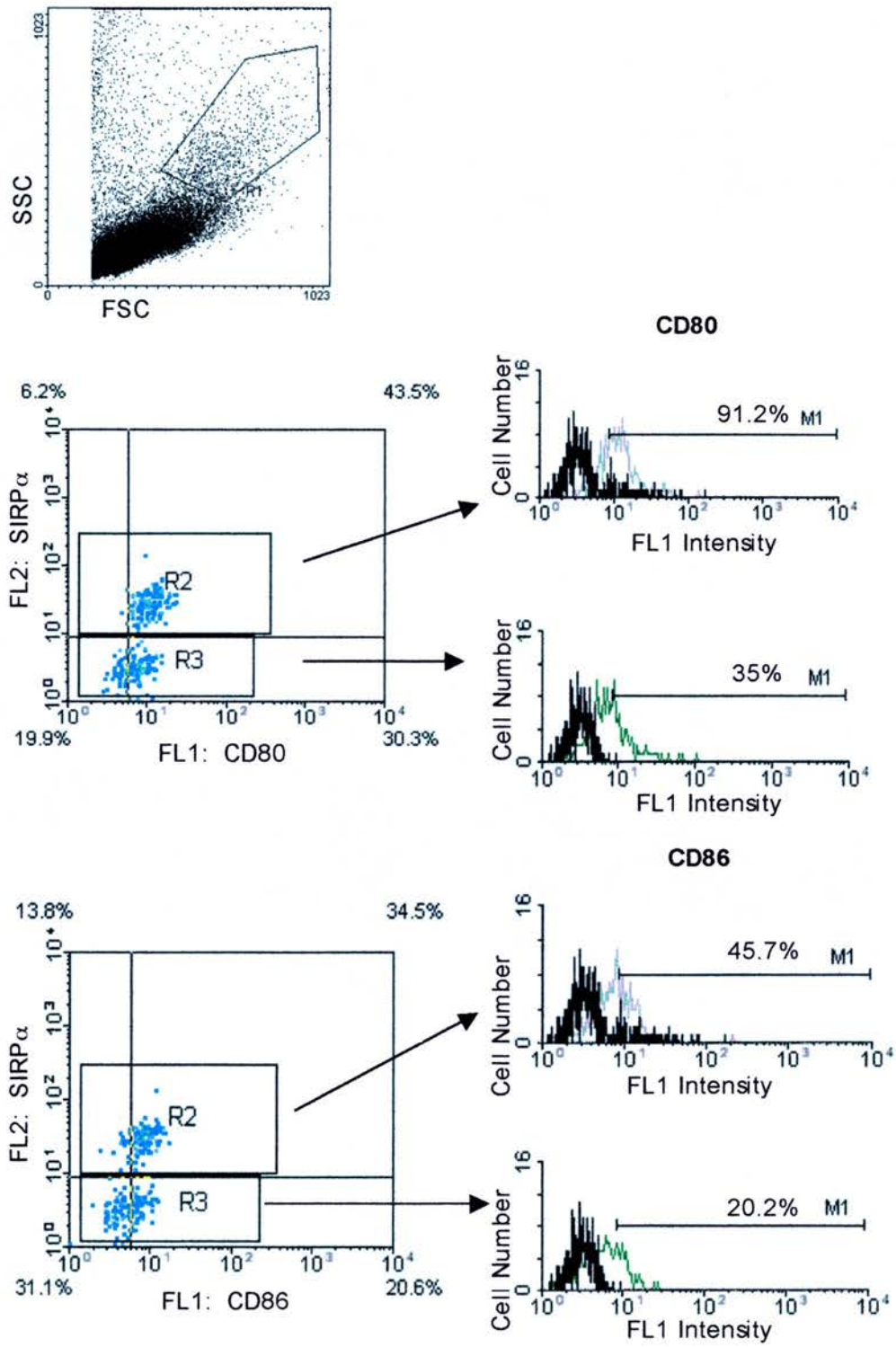
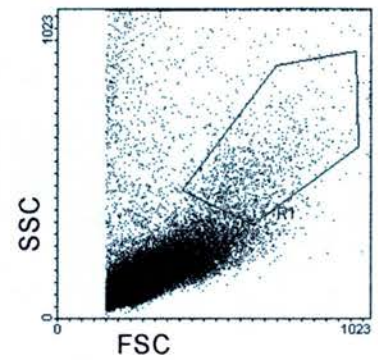
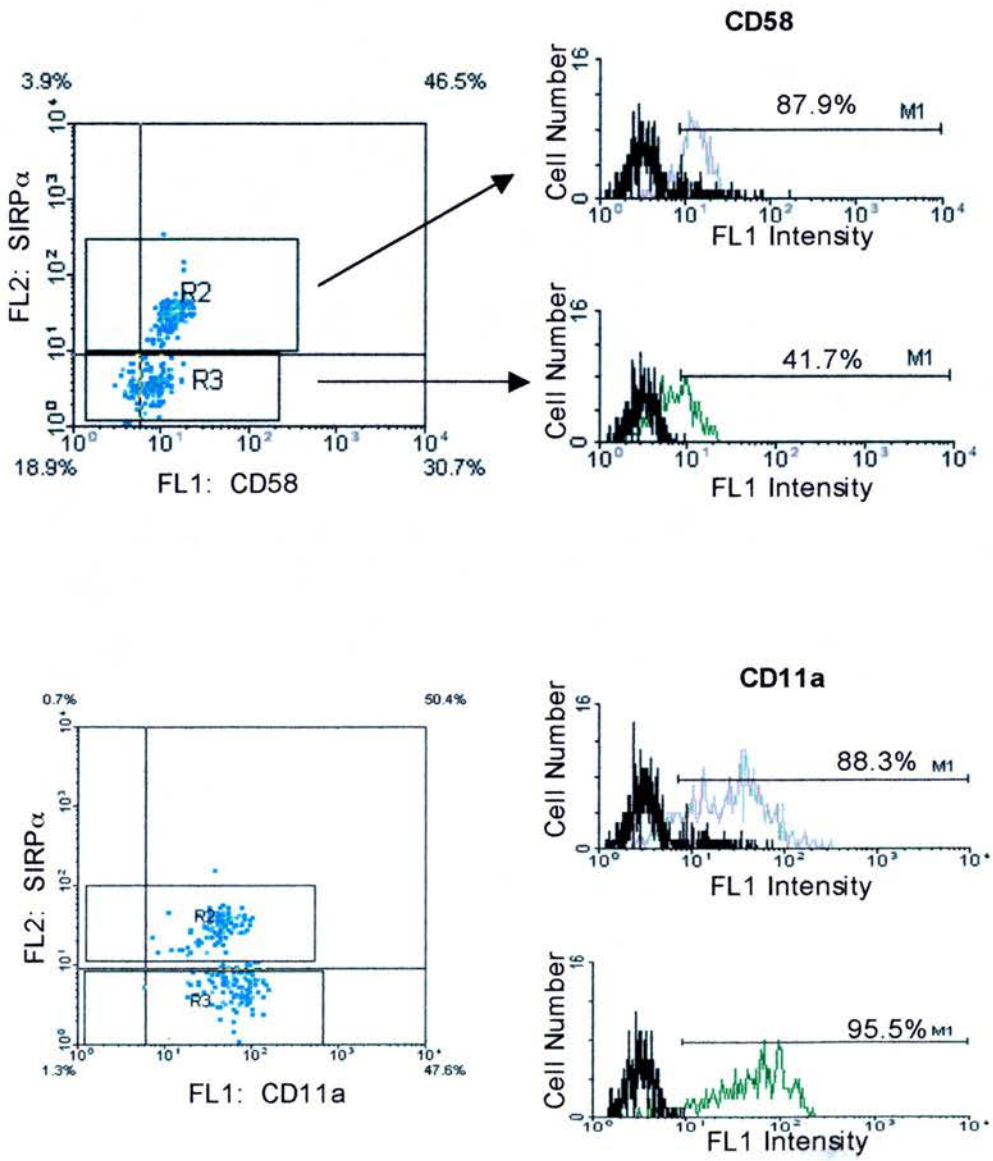

SSC
FSC
R1
CD80
6.2%
43.5%
19.9%
30.3%
FL2: SIRPα
FL1: CD80
R2
R3
Cell Number
91.2% M1
35% M1
FL1 Intensity
CD86
13.8%
34.5%
31.1%
20.6%
FL2: SIRPα
FL1: CD86
45.7% M1
20.2% M1

Figure 5.8


SSC
FSC
R1
1023
0


CD58
3.9%
46.5%
18.9%
30.7%
FL2: SIRPα
FL1: CD58
R2
R3
Cell Number
FL1 Intensity
87.9%
M1
41.7%
M1
CD11a
0.7%
50.4%
1.3%
47.6%
88.3%
95.5%

'spill over' from mAb reactivity on SIRPα$^-$ DCs (Fig 5.7). Conversely, 61.5% (± 2.8%) SIRPα$^-$ DC expressed CD45RA over 2-logs fluorescence ($p = < 0.001$).

CD16, which was expressed at negligible levels on DCs (2%, Section 5.2.4), was detectable on a minor portion of SIRPα$^+$ DCs (4.5% [± 1%]), and absent on SIRPα$^-$ DCs (1% [± 0.5%]). This pattern of expression on the DC populations was also demonstrated for CD206 and CD14 (Fig. 5.7). SIRPα$^+$ DCs contained significant proportions of cells positive for CD206 (56.9% [± 12.4%]) and CD14 (41.6% [± 10%]) compared with SIRPα$^-$ DCs that were 6.2% (± 1.4%) ($p = <0.02$) and 3.6% (± 1%) ($p = <0.02$) positive for CD206 and CD14 respectively.

SIRPα$^+$ DCs all expressed MHC II, CD40, CD80 and CD86 (as determined by FL1 shift; Fig. 5.7), whereas, while all SIRPα$^-$ DCs expressed MHC II, fractions did not express CD40, CD80 and CD86 (Fig 5.7). Intensity of CD40 and CD86 expression was similar between the two DC populations, CD40 staining of the whole population was significantly greater for SIRPα$^+$ DCs than SIRPα$^-$ DCs (99.3% [± 1.7%] vs. 77.5% [± 2.2%], respectively; $p = <0.001$), whereas CD86 staining was comparable (28.9% [± 8.5%] vs. 13.8% [± 4.1%], respectively; $p = >0.1$). Expression of CD80 differed between the DC population both in intensity (Fig 5.7) and proportions of staining, 72.5% (± 9.3%) SIRPα$^+$ DCs expressed CD80 above control levels, compared with 31.7% (± 1.7%) SIRPα$^-$ DCs ($p = < 0.05$).

CD58 and CD11a were expressed on a greater proportion of SIRPα$^+$ DCs than SIRPα$^-$ DCs, although this was not significant (Table 5.2).

5.2.7. Analysis of Constitutive Cytokine Expression by the SIRPα DC Populations.

In addition to determining the phenotype of lymph DCs, the 'psuedoafferent' lymphatic cannulation model was utilised to determine constitutive cytokine expression by DC populations undergoing migration to the lymph nodes. The following sections describe the enrichment of DCs from afferent lymph cells using a discontinuous multi-layered sucrose gradient, and purification of SIRPα populations by Fluorescence Activated Cell Sorting (FACS).

5.2.7.1 DC Enrichment

DC enrichment was achieved using the sucrose density media Optiprep ™ (Axis-Shield). A protocol optimised for ovine lymph was developed from published work describing the separation of monocytes from human blood (Graziani-Bowering, Graham and Filion, 1997). Details of the enrichment protocol are given in Section 2A.3. Lymph cells were separated by floatation from a fraction containing 28.6% optiprep, and a stepped gradient of 25% and 18.2 – 22% optiprep. Following centrifugation, two fractions of cells were evident at the media interphases. Cells were analysed by light microscopy, the upper fraction comprised cells of typical DC morphology possessing an irregular plasma membrane and were thus easily distinguishable from lymphocytes that are spherical and generally smaller and more dense than are DCs (Figure 2.1).

In order to achieve the optimal enrichment of DCs, a range of densities for the upper layer of Optiprep was tested. Decreasing the density of the upper gradient would selectively enrich for larger and less dense cells. Afferent lymph cells were separated using an upper layer consisting of 18.2%, 20% and 22% Optiprep, and cells at the media / HBS interphase analysed by flow cytometry, the results are presented in Figure 5.9. Cells with moderate - high FSC and SSC comprised 25%, 67% and 71% of cells isolated using 18.2%, 20% and 22% Optiprep, respectively (Figure 5.9). Enriched cells were characterised by surface marker expression, CD11c defined DC, CD11b defined monocytes / macrophages and B cells / activated T cells defined by reactivity to the VPM30 mAb. The data are summarised in Table 5.3. The proportion of DCs recovered increased by lowering the density of the upper layer of the Optiprep gradient, however at the lower density (18.2% Optiprep) monocyte / macrophage and B cell enrichment also increased. In the experiment presented above, the greatest enrichment of $CD11c^+$ DCs was achieved using an upper layer of 20% Optiprep, that represented 1% of the starting afferent lymph cells (2 x 52/100). In subsequent experiments, 20% Optiprep was used as the upper layer of density media for DC enrichment. Cell volumes of 2.5 ml or 10 ml at concentrations of $1 \times 10^6 - 2 \times 10^7$ cells / ml were separated with similar results.

Figure 5.9 Flow cytometric assessment of lymph DC enrichment. Afferent lymph cells were separated through a stepped gradient mixture of Optiprep™, using various concentrations for the upper layer. The low density cell fraction was collected and analysed by flow cytometry, the light scatter plots are shown. Gates were set for analysis, Gate R1 defines DCs. Gate R2 defines lymphocytes.

a) Before purification. DCs comprise 4% of the cells, lymphocytes 90%.

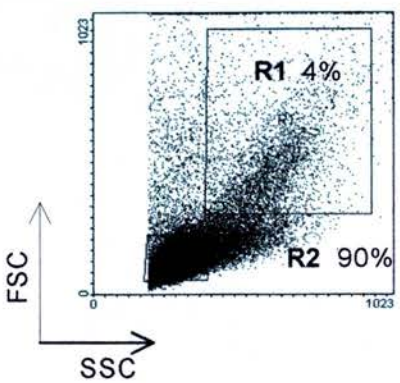
b) 22% Optiprep: DCs 25%, lymphocytes 60%.

c) 20% Optiprep: DCs 67%, lymphocytes 16%.

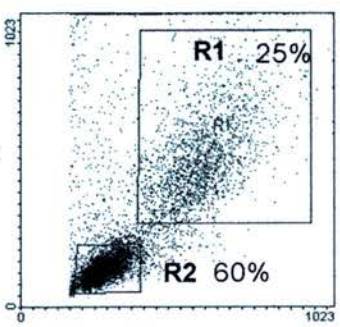
d) 18.2% Optiprep : DCs 71%, lymphocytes 10%.

Data are representative of two experiments using cells derived from one animal that was not used in other experiments presented in this Chapter.

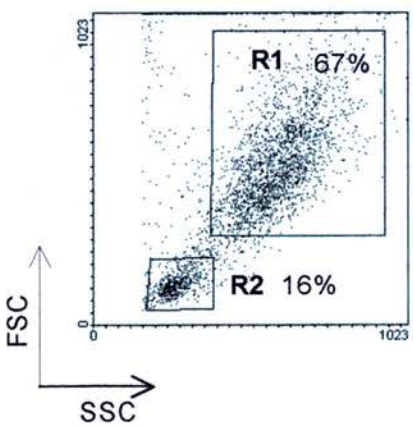
a) Afferent lymph cells



b) 22% Optiprep



c) 20% Optiprep



d) 18.2% Optiprep

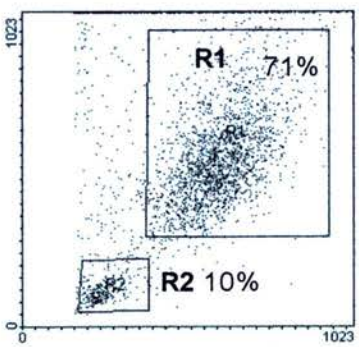


Table 5.3. Extent of DC enrichment using various concentrations of Optiprep™ density media

Upper Layer Optiprep (%)	DC (%)	Macrophages (%)	B cells (%)	% cell yeild[a]
22	26	1	8	5
20	52	2	32	2
18.2	62	3	32	0.5

[a] Percentage (%) cell yield is relative to the number of starting cells.

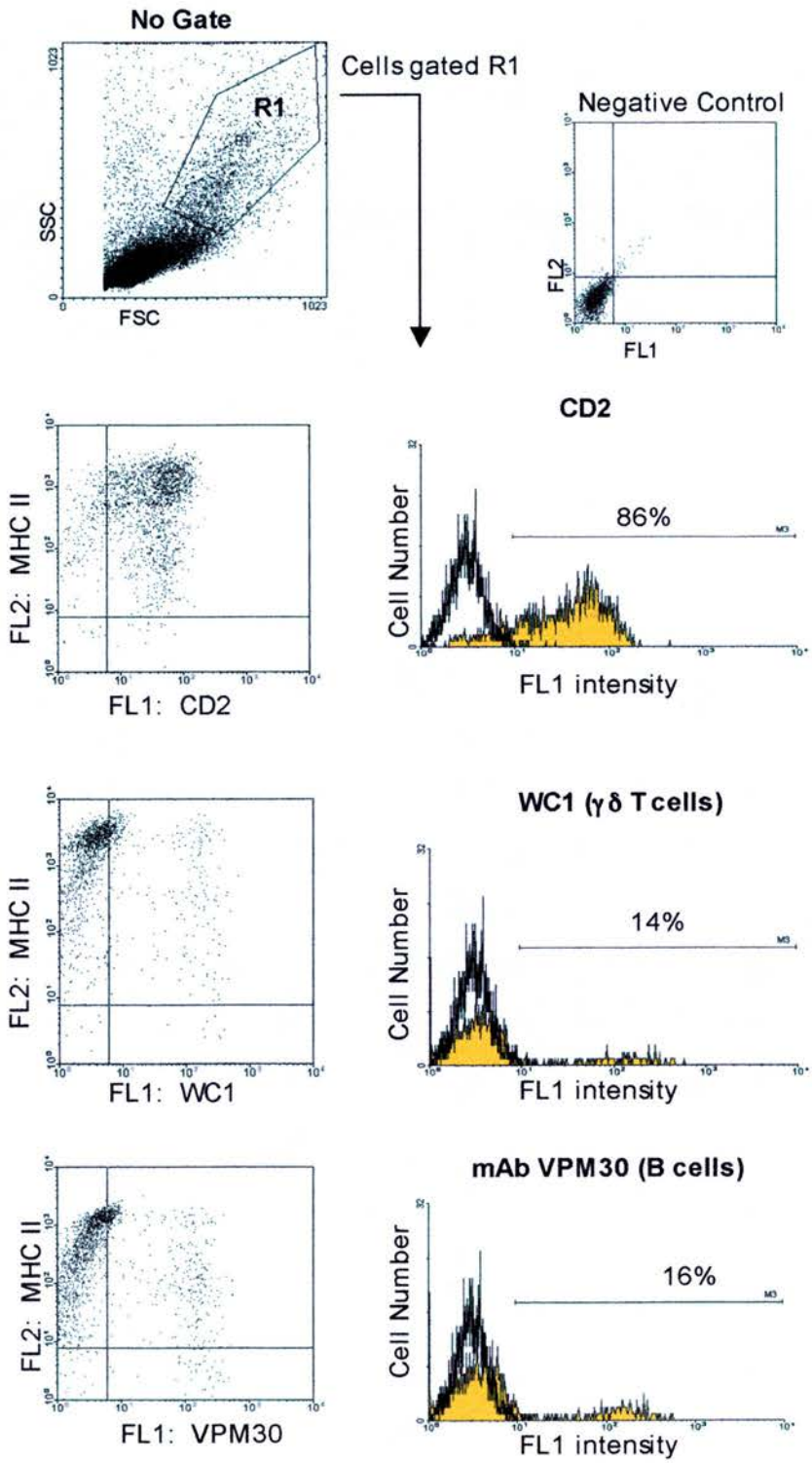
Cells were analysed following mAb staining and flow cytometry. Details of experimental procedures are given in the text.

5.2.7.2 Purification of DCs by Negative Selection was Unsuccessful

Purification of DC using cell surface markers and magnetic activated cell sorting (MACS) was investigated. MACS provides the advantages of high levels of purity of seperated cells, and allows the purification of cell populations from mixtures of $>10^8$ cells (Miltenyl Biotec). Negative selection of lymph DC was necessary because of: 1) the lack of DC-specific markers required at least two rounds of positive selection which may have resulted in a reduction of target cells collected, and low levels of purity; 2) Positive selection using specific mAb and magnetic beads would increase the likelihood of activating ligated receptors and modulating DC function. Therefore, the following mAb were titrated and used to deplete cell populations known to be present in afferent lymph: VPM30 (B cells / activated T cells); IAH-CC6 (anti-CD2 - αβ-T cells); IAH-CC15 (anti-WC1 - γδ–T cells); (see Table 2.1 for further details). Details of the protocol used for MACS separations are given in Section 2B.9. Cells eluted as the negative fraction were counted and analysed by flow cytometry (not shown). The number of cells recovered was approximately 10-fold lower than expected ie. 50% of DC enriched cells (Table 5.3). DCs were subsequently analysed by flow cytometry for reactivity to the depletion mAb. As shown in Figure 5.10, reactivity to mAbs VPM30 and IAH-CC15 was negligible. However, 86% of afferent lymph DCs expressed CD2 (Fig. 5.10). This

Figure 5.10 DC expression of 'lymphocyte markers'. Freshly isolated DCs were analysed by flow cytometry following labeling with anti-MHC II:biotin and streptavidin:PE, detected on the FL2 channel, and the mAb VPM30 (B cells / activated T cells), IAH-CC6 (anti-CD2) and IAH-CC15 (anti-WC1; γδ T cells), followed by anti:Ig:FITC conjugates, detected on the FL1 channel. 10 000 events were analysed per sample. A gate (R1) was set for DC analysis (FSC 400 – 1000; SSC 200 – 1000), as indicated on the dot plot. Negative controls were stained with serum or biotinylated serum and secondary conjugates. An example of negative control staining on FL1 vs. FL2 is shown. Dot plots show the extent of staining and level of MHC II expression, lymph DC express MHC II at > 3-logs fluorescence. Extent of marker expression is shown in the coloured histogram, negative controls are shown as the open histogram, percentage positive cells is shown with each profile.

Results are representative of two independent experiments with duplicate samples, on cells derived from Sheep 1 & 2.


No Gate
R1
SSC
FSC
Cells gated R1
Negative Control
FL2
FL1
CD2
FL2: MHC II
FL1: CD2
Cell Number
86%
FL1 intensity
WC1 (γδ T cells)
FL1: WC1
14%
mAb VPM30 (B cells)
FL1: VPM30
16%

was surprising because there was no published evidence of significant CD2 expression on DCs, and although CD2 expression by sheep DCs had been demonstrated by flow cytometry, the authors proposed that mAb reactivity may be due to absorption of the molecule (Bujdoso et al., 1989). The mAb specific for αβ-T lymphocyte-restricted markers, (such as CD28 or CD3) were not available during this study. Therefore the negative selection of DC from afferent lymph cell populations was not possible. The following section describes the isolation of DC populations using FACS.

5.2.7.3 Purification of DC Populations by FACS

$WC6^+$ $SIRP\alpha^+$ and $WC6^+$ $SIRP\alpha^-$ DCs were purified using FACS. Following density media enrichment as described in Section 5.2.7.1, DCs were stained for WC6 and SIRPα, and live cells sorted using a FACS Vantage, details are given in Section 2B.8.4. A representative experiment indicating the gate criteria for cell sorting is shown in Figure 5.11. The cell fractions were analysed following sorting and the $SIRP\alpha^+$ and $SIRP\alpha^-$ cell fractions consistently >98% and 96% pure respectively, based on the presence or absence of SIRPα (Figure 5.11).

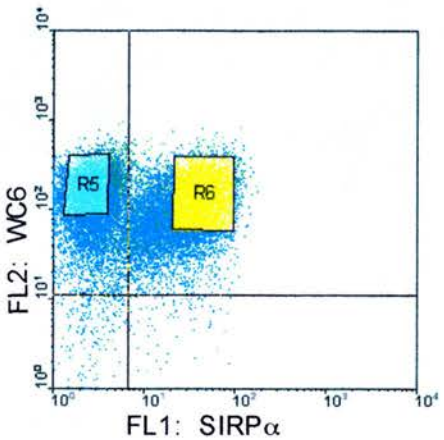
5.2.8 Morphology of the $SIRP\alpha^+$ and $SIRP\alpha^-$ DCs

DCs separated into $SIRP\alpha^+$ and $SIRP\alpha^-$ populations were examined following cytocentrifugation and staining with Giemsa stain, as described in Section 2A6. Representative images are shown in Figure 5.12. The $SIRP\alpha^+$ cells were relatively large, containing spikey processes. In contrast, $SIRP\alpha^-$ DCs were generally smaller with blunt pseudopodia or veiled edges evident on a number of the cells. These morphological distinctions were not entirely confined to the sub-populations, and cells of intermediate morphology were evident in both fractions.

5.2.9 Constitutive Cytokine Expression by DC Sub-populations

To ensure gene expression was not modulated during collection and processing of the cells, Actinomycin D was added to the afferent lymph collection bottles to give a final concentration of approximately 1 μg/ml. Buffers contained

Figure 5.11 Fluorescent activated cell sorting DC SIRPα sub-populations. DCs were stained sequentially with anti-SIRPα then FITC anti-Ig conjugate, followed by biotinylated WC6 and streptavidin-PE. Staining was detected on the FL1 and FL2 channels, respectively. The dot plot shows DC expression of WC6 and SIRPα. Cells were gated for sorting: R5 defines the SIRPα$^-$ population; R6 the SIRPα$^+$ population. Sorted cells were analysed for SIRPa expression and are presented in the histograms, the negative cell fraction is shown in blue, the positive in the open profile. This type of analysis demonstrated the cell fractions were consistently 96% and 98% pure respectively, defined by SIRPα staining and scatter profiles. Data is representative of ten separate experiments, performed on cells from Sheep 1 & 2.



SIRPα

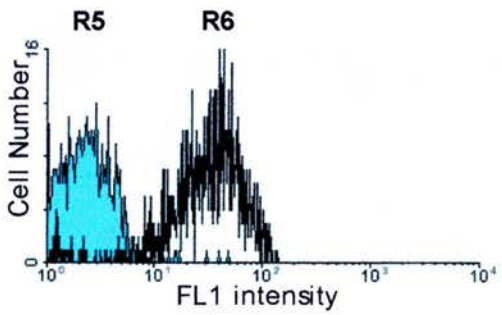


Figure 5.12 Morphological distinctions of SIRPα$^+$ and SIRPα$^-$ DCs. DCs were enriched from lymph by density media centrifugation and separated into SIRPα positive and negative fractions by FACS. Freshly isolated cells were assessed microscopically following Giemsa staining.

a) SIRPα$^+$ DCs display multiple short, fine processes.

b) SIRPα$^-$ DCs possess several long pseudopodia

These morphological distinctions are not entirely distinct, and cells of intermediate morphologies were found in each fraction.

a) SIRPα$^+$

b) SIRPα$^-$

1 μg/ml Actinomycin D. Actinomycin D inhibits transcription by disrupting the DNA helix (Chen, Liu and Patel, 1996). The concentration of Actinomycin D used in these experiments was determined to be non-toxic to cells (by trypan blue exclusion).

DCs were purified and SIRPα$^+$ and SIRPα$^-$ cells isolated as described in Section 5.2.7. Total RNA was isolated from DCs collected over 24 hour periods and analysed. The advantage of analysing individual samples was to obtain a cohort of samples from individual animals. The disadvantage, however, was the low yield of extracted RNA, 2-3 x 10^5 SIRPα$^+$ / SIRP$^-$ DCs yielded less than 250 ng total RNA. The cytokine RPA (Chapter 4) was not suitable for the analysis of such low numbers of cells. Therefore rtPCR was utilised detect IL-6, IL-10, IL-12 p40 and IL-18 transcripts expressed by the SIRPα$^+$ / SIRPα$^-$ DC sub-populations. This technique was employed because of its specific nature and ability to detect mRNA transcripts in low abundance. Regrettably, time constraints of the project did not allow for establishing a real-time PCR protocol, which would allow the quantification of cytokine mRNA transcripts utilising internal controls. Detection of mRNA by standard RT-PCR gives an indication of transcription of a particular gene, but no information on the relative levels of expression and indeed the production of a functional protein. This method is not quantitative and correlations of transcript abundance between the two cell populations cannot be made. Accordingly these experiments were carried out to confirm the presence or absence of cytokine transcripts in freshly isolated DC populations.

5.2.9.1 Sheep DC SIRPα Populations Exhibit Differential Cytokine Expression.

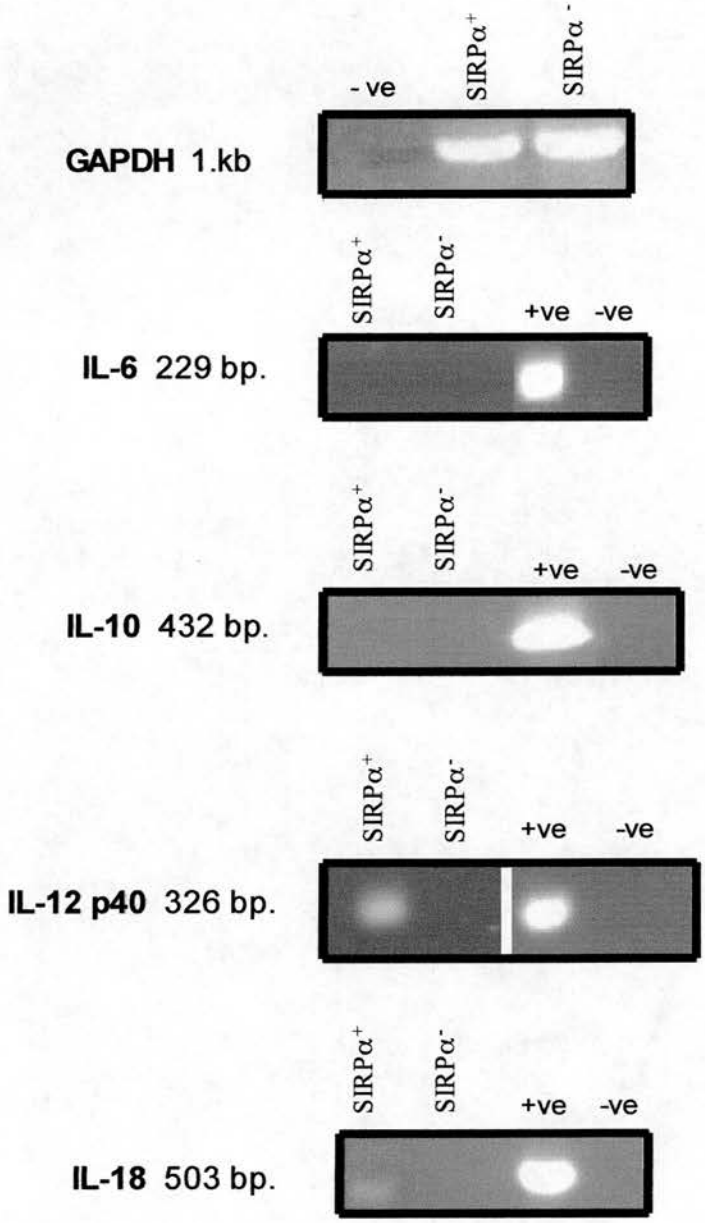
RNA extracted from SIRPα$^+$ and SIRPα$^-$ DCs (Section 2C.1) was treated with DNase I. Samples were then concentrated by precipitation and centrifugation under a vacuum, and re-suspended in 15 μl nuclease-free H_2O. 5 μl of the RNA sample from each cell population were pooled and analysed for genomic DNA content (Section 2C.2). These were consistently negative (not shown). Positive controls were 10 ng plasmid DNA containing cytokine cDNA, amplified for 30 cycles using the appropriate PCR primers. Template DNA was omitted to control for

reagent contamination. PCR reactions were standardly optimised for [$MgCl_2$] and dNTPs.

All primers were checked for specificity by rtPCR using LPS stimulated macrophage RNA as template (not shown). Single products were purified, cloned and sequenced (Sections 2C.4 and 2C.5). Analysis using GAP software (GCG 10) confirmed the specificity of the primers. The IL-18 primers were confirmed for specificity as described in Chapter 3.

Figure 5.12 shows the amplification products of rtPCR analysis of SIRPα^+ and SIRPα^- DC RNA. These results are representative of two independent cell collections from Sheep 2. PCR reactions were performed in duplicate. GAPDH amplicons were not detected during rtPCR analysis of RNA collected from Sheep 1, and were therefore not utilised further. Similar levels of GAPDH PCR products were evident in the SIRPα^+ and SIRPα^- DC RNA preparations derived from Sheep 2, indicating similar amounts of intact cellular RNA. IL-12 p40 transcripts were easily detectable from less than 250 ng total RNA isolated from SIRPα^+ DCs, and were absent in SIRPα^- DCs. Similarly, IL-18 was detected in only the SIRPα^+ DC sample, albeit at low levels. IL-6 and IL-10 transcripts were not detected in either DC population.

Figure 5.13. Analysis of constitutive cytokine expression by SIRPα$^+$ and SIRPα$^-$ DCs. Freshly isolated lymph DCs were purified according to the presence or absence of SIRPα as described in Section 5.2.7. RNA was extracted, concentrated and subject to RT-PCR analysis using 40 cycles of amplification utilising primers for GAPDH, IL-6, IL-10, IL-12 and IL-18. Positive control reactions contained plasmid DNA containing cytokine cDNA as template and were amplified for 30 cycles, these are indicated with (+ ve), cDNA template was omitted in negative controls (-ve). SIRPα positive cells expressed transcripts for IL-12 p40 and IL-18. No cytokine products were detected in the SIRPα negative cells. Data is representative of duplicate PCR reactions from two independent cDNA synthesis reactions, using DCs derived from Sheep 2.


- ve
SIRPα+
SIRPα -
GAPDH 1.kb
SIRPα+
SIRPα-
+ve
-ve
IL-6 229 bp.
SIRPα+
SIRPα-
+ve
-ve
IL-10 432 bp.
SIRPα+
SIRPα-
+ve
-ve
IL-12 p40 326 bp.
SIRPα+
SIRPα-
+ve
-ve
IL-18 503 bp.

5.3 DISCUSSION

Given the overall heterogeneity of DCs, it is accepted that the identification of DCs relies on a combination of characteristics including: 1) morphology, with existence of dendrites and / or long psuedopodia; 2) surface phenotype, demonstrating expression of molecules involved in T cell activation; 3) T stimulatory capacity (Steinman, 1991). The SIRPα DC populations fulfil the first two of these criteria, and a previous study by our group (Bujdoso et al., 1989), demonstrated that ovine lymph DCs were potent stimulators of allogeneic and autologous T cells, therefore fulfilling the latter DC characteristic.

5.3.1 Biological Consequence of Lymph DC Population Phenotype

Both SIRPα$^+$ and SIPRα$^-$ DCs expressed MHC II over 2-logs fluorescence, expressed similar levels of CD11a, and all SIRPα$^+$ DC and most SIRPα$^-$ expressed CD58. MHC II interactions with antigen specific TcRs constitute 'signal one' in DC-T cell interactions. Initial interactions during clustering with antigen-specific T cells are in part mediated by CD11a and CD58 (Selvaraj et al. 1987; Breitmeyer et al., 1987). Expression of MHC II, CD11a and CD58 indicates both ovine DC populations were phenotypically equipped to participate in antigen presentation to lymphocytes.

Lymph DC subpopulations displayed a dichotomy of expression of co-stimulatory molecules required for sustaining T cell activation (Fig. 5.8). Based on histogram staining shifts on FL1, all SIRPα$^+$ DCs expressed CD40, CD80 and CD86, whereas most SIRPα$^-$ did not express CD86, and levels of CD40 and CD80 were significantly lower on the SIRPα$^-$ DC populations as a whole. However, expression levels of CD86 were similar on the DC populations, and that CD86 has prominent T-cell activation on DC (Caux et al., 1994; Inaba et al., 1994) indicates that prior to cognate interactions with T cells, the populations have similar capabilities for constitutively activating clustered T cells in the lymphatics. T cells activate DC via CD40, leading to increased production of IL-12, IL-1, TNFα, and increased expression of CD80 and CD86 (Cella et al., 1996; Grewal et al., 1996; Ridge, Di

Rosa and Matzinger, 1998). That a fraction of SIRPα$^-$ DCs did not express CD40 suggests these cells require additional maturation signals to express CD40, initiate cognate interactions with T cells and produce IL-12. Whether the CD40$^-$ SIRPα$^-$ DCs were also negative for CD80 and CD86 was not established in this study, and requires 4-colour flow cytometry to determine that.

Significant differences in the level of expression of molecules involved in pathogen recognition and antigen uptake were found between the DC populations. Expression of CD206, CD16 and CD14 was mainly confined to the SIRPα$^+$ DC population. Levels on the SIRPα$^-$ DCs were either low (CD206) or absent (CD16, CD14) (Fig. 5.8). CD14 functions as a co-receptor of TLR4 and is required for LPS responsiveness (Haziot et al., 1996). Moreover, SIRPα$^+$ DC, but not SIRPα$^-$ DC express TLR4 (K. Mathews, Edinburgh University; personal communication), suggesting that the former may be more responsive to LPS and IL-12 priming than the latter.

CD206 and CD16 promote antigen loading into MHC II-rich lysosomes, resulting in highly efficient MHC II loading and presentation (Inaba et al., 1997). Ligation and activation of CD16 and CD206 or DC maturation induced by LPS, CD40 or the cytokines IL-1 and TNFα results in the down-regulation of CD16 and CD206 expression, and a reduced capacity for antigen uptake (Sallusto et al., 1995; delaSalle et al, 1997). These processes switch the DC from being highly efficient at antigen uptake, to stable loading of MHC I and II molecules and presentation at the cell surface of exogenous antigenic peptides. Low level CD16 and CD206 expression on SIRPα$^+$ DC suggests that the tissue DCs from which this population is derived expressed CD16 and CD206. That pathogen / antigen recognition receptors present on a population of migrating ovine DCs may have functional consequences, such as enhancing basal levels of cytokine expression, (discussed in further detail in Chapter 6), may indicate SIRPα$^+$ DCs are related to DC populations described in mice and humans (Section 5.3.6).

CD11c is expressed on a major fraction of DCs in murine and human peripheral lymphoid organs (Anjuere et al., 1999), which appears to be the situation for sheep as 83.5% (± 1.8%) DCs migrating from cutaneous tissue expressed CD11c (Fig. 5.4). SIRPα$^+$ DCs expressed intermediate levels of CD11c, whereas SIRPα$^-$

DCs contained fractions of DCs with low level or undetectable CD11c expression (Fig. 5.8). CD11c forms the α–subunit of complement receptor-4 (CR4, CD11c/CD18), and promotes the phagocytosis of activated complement component 3b (iC3b)-opsonised products and contributes to the uptake of apoptotic cells by splenic marginal zone DCs (Morelli et al., 2003). The function of ovine CD11c/CD18 has not been established, and it would be important for tolerance-directed therapies to determine if ovine DC utilise CD11c-dependant mechanisms for the uptake of apoptotic cells. SIRPα$^+$ DCs share certain analogies with LCs and their lymphoid counterpart IDC (Section 5.3.6.1). LCs comprise a significant proportion of marginal zone DC in lymph nodes (Anjuere et al., 1999), and, therefore, may have a role in the uptake and presentation of cytoplasmic antigens in cells undergoing apoptosis in the lymph nodes. Re-presentation of antigens by IDCs forms an indirect mechanism of antigen presentation *in vivo*, and that SIRPα$^+$ lymph DC population could perform this function in sheep could be hypothesised based on conclusions discussed in Section 5.3.6.1. Tracking studies using fluorescent-labeled antigens or cells administered sub-cutaneously could be used to investigate this in future studies.

WC6 was used as a marker for DCs in this study, so it was unexpected that the two DC populations differed significantly in WC6 expression: 92% (± 0.1%) SIRPα$^+$ DCs expressed WC6, whereas 77.3% (± 4.7%) SIRPα$^-$ DCs displayed heterogeneous expression of WC6. Thus, 16% of afferent lymph DCs (mod – high FSC vs. SSC [Budjoso et al., 1989]) were WC6$^-$, and further experiments are required to determine if this cell fraction does indeed represent functionally active DCs - such as displaying a high capacity for stimulating T cell proliferation *in vitro*. The SIRPα$^-$ WC6$^-$ cells however have phenotypic characteristics of DCs, such as moderate-high FSC vs. SSC (Bujdoso et al., 1989) and MHC II expression over 2-logs fluorescence (Hopkins et al., 1985). This highlights that caution is required when characterising ruminant DC populations on the basis of WC6 expression alone, and more than one property for identifying DCs *ex vivo* is advantageous (as was used in this study). Such an example was demonstrated in the mouse system, as the characterisation of DCs in secondary lymphoid organs based entirely on CD11c expression resulted in plasmacytoid DCs not being identified (Ardavin, 2003).

Both DC populations reacted with antibodies against CD4 and CD8 antigens (Fig. 5.6). DCs have been shown to express both CD4 & CD8 (Vremec et al., 2000) and not display reactivity by the passive uptake from T cells. Murine DC subsets are classified by expression of the CD8αα homodimer, and as the reactivity of the anti-CD8 mAb SBU-T8 has not been classified to a particular chain of CD8, whether this mAb is useful for determining DC lineages in sheep is not known. As discussed in Section 5.2.4., it is likely that proportions of the CD4 and CD8 staining represented mAb reactivity with activated lymphocytes that spontaneously form clusters with DCs in afferent lymph, that were evident following antibody staining (Fig 5.6). CD11a and CD58 expressed by both DC populations could contribute to constitutive DC:lymphocyte clustering *in situ*.

CD45RA, the high molecular weight isoform of the common leucocyte antigen CD45, was expressed by a major fraction of the SIRPα$^-$ DC sub-population, whereas SIRPα$^+$ DCs did not express this antigen. Human pDCs and DC/NK cell precursors are the only DC subsets reported to express CD45RA (Canque et al., 2000; Liu et al., 2001), which may be relevant for making comparisons with ovine lymph DC subsets, as discussed in Section 5.3.6. CD45 is a transmembrane tyrosine phosphatase, its primary molecular targets are the Src-family kinases (Thomas and Brown, 1999). CD45 negatively regulates cytokine and interferon receptor mediated activation (Irie-Sasaki et al., 2001), and is important in down regulating kinase activity during integrin-mediated adhesion in macrophages (Burns et al., 1994). The differential expression of CD45RA on DCs suggests functional consequences. Because of the role of CD45 in the regulation of cellular events during adhesion, it would be interesting to investigate the function of CD45RA$^+$ compared to CD45RA$^-$ DCs during DC:T cell interaction.

5.3.2 SIRPα DC Populations

The data indicate that ovine SIRPα$^-$ DCs were markedly more heterogeneous than SIRPα$^+$ DCs. SIRPα$^+$ DCs expressed MHC II, CD40, CD80, CD86, CD11c and WC6 as a single population. Expression of CD16, CD14, CD206 were low or absent on SIRPα$^+$ DCs, and may have been modulated (Section 5.3.4). SIRPα$^+$ DCs contained cells with two levels of expression of CD58 and CD11a. Conversely,

SIRPα$^-$ DC expression of MHC II ranged from intermediate to high, and this DC population contained proportions of cells that were negative for CD58, CD40, CD80 and CD86. Expression of CD11c and WC6 were heterogeneous or absent on SIRPα$^-$ DCs. Only CD11a expression produced a unimodal histogram. It appears therefore, that both SIRPα$^+$ and SIRPα$^-$ DCs may comprise cells at different stages of maturation, or of different sub-populations – particularly SIRPα$^-$ DCs. This observation differs with that reported for cattle DCs. Bovine lymph SIRPα$^-$ DCs were reported to be homogenous, whereas cattle SIRPα$^+$ DCs were heterogeneous (Howard et al., 1997). In that study the cattle DC populations were not assessed for markers investigated in the current study, and heterogeneous expression of CD5, CD21 and WC10 indicated bovine SIRPα$^+$ DCs were a mixed population. These molecules were not investigated in this study, and the disparity between ovine and bovine DCs may be due differences in the molecules analysed. CD11a expression however, differed markedly between cattle and sheep DCs: CD11a was expressed only by SIRPα$^+$ DCs in cattle (Howard et al., 1997), whereas SIRPα$^+$ and SIRPα$^-$ DCs in ovine lymph expressed CD11a at similar levels. More data is required on DCs from both species if conclusions are to be made regarding their relativity to each other.

5.3.3 Animal Variation

Patterns of cytokine expression were determined in cells from an individual animal (Sheep 2). DCs from Sheep 2 presented a phenotype representative of three animals, and may therefore be also representative for determining cytokine expression. However, these experiments should be repeated in an additional two animals to confirm constitutive cytokine mRNA patterns in SIRPα DC populations. MHC II expression differed significantly in Sheep 3, as did DC phenotype. This could reflect actual individual differences, or physiological differences such as infection. Signals of infection, such as lymph leucocytosis (determined by flow cytometric scatter plots), or oedema around the surgical site were not evident. However, this cannot be discounted. Inflammatory cytokines produced in response to infection would induce the recruitment and activation of blood leucocytes into the tissue, and afferent lymph. Activated lymphocytes express WC6 (Dutia, Ross and

Hopkins, 1993) and increase FSC SSC readings, and could have been included in the DC gate during analysis of cells from Sheep 3.

5.3.4 Maturation Status of the Ovine DC Sub-populations

Due to high-level expression of MHC II, and CD40, CD80 and CD86, SIRPα^+ DCs resembled mature DCs. SIRPα^- DCs, however, display lower expression of MHC II and co-stimulatory molecules and therefore resembled a more immature DC. On the other hand, SIRPα^+ DCs also expressed CD14, CD16 and CD206, which represents an immature phenotype in mice (Sallusto et al., 1995). It may however, be presumptuous to conclude that expression of CD14, CD16 and CD206 on sheep DCs indicates an immature phenotype, because porcine monocyte-derived DCs (cultured with GM-CSF and IL-3) retained CD14 and CD16 expression after maturation *in vitro* (Carrasco et al., 2001). Overall therefore, high homogeneous expression of molecules involved in antigen presentation and activation of T cells is a strong indicator that SIRPα^+ DCs were at a mature stage of differentiation, which could relate functionally to increased T-stimulatory potential.

5.3.5 Cytokine Production

The biological relevance of constitutive expression of IL-12 and IL-18 by migrating DCs *in vivo* is not fully understood. IL-12 and IL-18 are expressed constitutively by LCs (Kang et al., 1996; Stoll et al., 1998), and mediate rapid Th1-mediated responses in the skin (contact hypersensitivity) (Wang et al., 2002). Not surprisingly, IL-12 and IL-18 transcripts are detected at basal levels in mouse lymph nodes (Stoll et al., 1998; Wang et al., 2002). However the release of biologically active IL-12 and IL-18 are tightly regulated (Ghayur et al., 1997; Trinchieri, 1998). The IL-12 molecule is a heterodimer consisting of two disulphide-bonded subunits, IL-12 p40 and IL-12 p35, encoded on separate genes, that must be expressed at the same time to produce the bioactive molecule (Gately et al., 1998; Trinchieri, 1998). In DCs, maturation signals induce IL-12 p40 production, and CD40 triggering results in IL-12 p35 induction (Schulz et al., 2000). Expression of IL-12p40 in migrating ovine DCs provides further evidence SIRPα^+ DC are mature. Production of high levels of IL-12 p70 and the generation of Th1 cells requires cognate CD40

interactions (Cella et al., 1996; Koch et al., 1996), which occur transiently during the activation of antigen-specific T cells (Macatonia et al., 1995; Koch et al., 1996).

Production of IL-18 is regulated by endogenous pathways, requiring the cleavage of biologically inactive proIL-18 by caspase-1 (ICE) (Ghayur et al., 1997; Gu et al., 1997). Expression of IL-12p40 and IL-18 by SIRPα^+ DCs indicates the cells are primed for production of these cytokines, and implies they may mediate Th1-biased responses. Low-level IL-12 and IL-18 production may also promote the activation of T cells clustered with DC in afferent lymph. In general, the production of IL-12 by DCs produces a Th1-type response (production of IFNγ) (Macatonia et al., 1995; Hilkens et al., 1997), and IL-18 enhances Th1 cell development (Ushio et al., 1996).

The absence of IL-12, and the presence of IL-6, IL-10 and IL-4 contribute to a Th2-type response (production of IL-4 and IL-5) (Paul and Seder, 1994; Rincon et al., 1997), and the suppression of IL-12 production (Trinchieri, 1998). IL-10 and IL-6 were not detected in either DC population, indicating there were no effects on IL-12 at the basal level of expression in SIRPα^- DCs. In addition, lack of cytokine detection in SIRPα^- DCs infers that this population were not biased to promote the development of a particular type of T helper cell.

5.3.6 Ovine Lymph SIRPα DC Populations Have Similarities With Murine DC Populations

5.3.6.1 SIRPα^+ DCs

Cells in the epidermis and dermis express SIRPα (Adams et al., 1998), indicating that SIRPα^+ lymph DCs are derived from these locations. Phenotypically, ovine SIRPα^+ DCs resembled interstitial DCs (IDCs) of lymph node T cell areas. IDCs express high levels of MHC II, CD40, and CD80, and low levels of FcRs, and derive from epidermal LCs (Anjuere et al., 1999; Salomon et al., 1998; Henri et al., 2001). IDC represent 65% of lymph node DCs (Salomon et al., 1998), which is comparable to the proportion of SIRPα^+ DCs in ovine afferent lymph (71.2% ± 2.5%). Moreover, LCs express IL-12 and IL-18 constitutively (Kang et al., 1996; Wang et al., 2002). Therefore, surface phenotype and constitutive cytokine

expression by SIRPα$^+$ DCs suggest that this population derives, at least in part, from epidermal LCs. Following cognate interactions with antigen-specific T cells, LCs induce strong Th1-biased development (Everson et al., 1996; Kang et al., 1996; Stoll et al., 1998). That SIRPα$^+$ DCs may be the migratory stage of LCs, be equivalent to IDC of murine LNs, and express IL-12 and IL-18 transcripts, strongly suggests that this population would have potent T-stimulatory capacity *in vivo*, and may be important for cell mediated immunity against intracellular pathogens. Further evidence to support this hypothesis is required; such as *in vivo* activation with maturation stimuli - such as microbial adjuvants or cytokines, and the cytokine responses of SIRPα$^+$ DCs determined. If indeed this population produces high levels of IL-12, and induces high levels of IFNγ production by T cells *in vitro*, strategies for targeting this population in vaccination regimens could be developed.

5.3.6.2 SIRPα$^-$ DCs

A major fraction of SIRPα$^-$ DCs expressed CD45RA (61.6% ± 2.8%). Human pDC are the only DC population reported to express CD45RA (Liu et al., 2001). Further phenotypic similarities can be made with fractions of ovine SIRPα$^-$ DCs and murine lymphoid B220$^+$ pDCs (Asselin Paturel et al., 2001) (which may more closely represent lymph DCs). These include, low levels of CD40 and CD80 expression; low and heterogeneous MHC II and CD11c expression, lack of CD86, CD14 and other myeloid markers. This study did not determine the phenotype of sub-populations within SIRPα$^-$ DCs. In addition, there is no functional data at this time to determine the biological functions of ovine SIRPα DCs *in vivo*. Therefore, links between ovine DCs and murine DCs are tentative, but still, intriguing. Murine pDCs characteristically produce high amounts of type I interferons (Asselin Paturel et al., 2001), and have tolerogeneic effects on T cells *in vitro* (Martin et al., 2002; Bilsborough et al., 2003), leading the authors to conclude they may function to support peripheral tolerance *in vivo*. Interestingly, SIRPα$^-$ DCs in rat lymph have also been hypothesised to process and present endogenous antigen, and function to support peripheral tolerance (Huang et al., 2000). Rat lymph DCs (Liu et al., 1998) share similarities with ovine lymph DC populations. Both SIRPα$^+$ and SIRPα$^-$

subpopulations express CD11c, MHC II and CD80, and the DC populations are morphologically equivalent, in that SIRPα$^+$ DCs are more 'spikey' in appearance than SIRPα$^-$ DC that appear 'frilly'. However, like with bovine DCs populations, more evidence is required to determine whether ovine and rat DCs are equivalent.

Evidence in cattle however, suggests SIRPα$^-$ DCs are not plasmacytoid in nature. Following activation with virus or dsRNA *in vitro*, bovine SIRPα$^-$ DCs did not produce type I interferons after activation with virus, as determined by a gene reporter assay (Stephens et al., 2003). However, the SIRPα$^-$ DC populations in sheep and cattle appear different (Section 5.3.2), and SIRPα$^-$ cattle DC were reported to have a mature phenotype (Howard et al., 1997). Whether the ovine SIRPα$^-$ DCs that co-express CD40, CD80 and CD86 are a separate cell fraction that are phenotypically more equivalent to cattle SIRPα$^-$ DCs remains to be elucidated. Moreover, SIRPα$^+$ and SIRPα$^-$ cattle DCs both express IL-12 and IL-18 (Stephens et al., 2003), whereas these cytokines were only detectable in sheep SIRPα$^+$ DC and not SIRPα$^-$ DCs. These may reflect actual differences between these two closely related species, or differences in lymphatic cannulations, collection of cells, and methods of analysis. In the study of Stephens et al. (2003) DCs were collected in the acute stages following surgery, when injury induced danger signals, such as HSPs could activate the DC (Basu et al., 2000), and skew resting cytokine responses. In addition, the bovine DCs were analysed following cryopreservation that could affect both cytokine and surface marker profiles. The ovine DCs described in this project were analysed immediately following 24 hour collections from chronically cannulated sheep. This was limiting for the availability of sample (for example mRNA), however it was highly desirable for the analysis of constitutive biological characteristics of lymph DCs migrating to the lymph nodes under steady-state conditions. Therefore, because of differences in experimental procedures, cattle and sheep lymph DC populations cannot be directly compared. Further experiments are required to determine if ovine SIRPα$^-$ DCs have pDC characteristics, such as high levels of IFNα production following stimulation with virus. Plasmacytoid DCs are suppressive in nature, and have been identified as ideal candidates for DC based therapies against autoimmune diseases (Kuwana, 2002), such as multiple sclerosis

and type I diabetes. Therefore investigations into these cells have great therapeutic impacts on human health and disease, and the requirement of knowledge of the physiological characteristics of these cells support the use of *ex vivo* systems such as ovine lymphatic cannulation.

5.3.7 Signal Regulatory Protein-α (SIRPα)

The presence of an ITIM in the SIRPα cytoplasmic tail (Brooke, Parsons and Howard, 1998) suggests it plays a role in modulating signalling pathways in lymph DCs. Human SIRPα functions in both a positive and negative manner (Kharitonenkov et al., 1997), thus, SIRPα presence on sheep DCs may regulate their function. SIRPα is particularly effective at regulating IL-12p70 release following the activation of human monocyte-derived DCs with *Staphylococcus cowan* extract (SAC) or LPS (Latour et al., 2001). SIRPα and CD47 interactions have been proposed to act as a negative feedback mechanism during T cell activation (Waclavicek et al., 1997), which may be due to control of IL-12 release. Interestingly, SIRPα is expressed on the ovine DC population that is 'primed' for IL-12 release (see below).

The role of SIRPα on un-stimulated DCs is less clear. SIRPα may play a role in the activation of T cells. Interactions with its ligand CD47 have been shown to enhance T cell proliferation in a CD3-dependant manner (Ticchioni et al., 1997; Reinhold et al., 1997; Waclavicek et al., 1997), enhancing low level thresholds of anti-CD3 stimulation (Reinhold et al., 1997). Acting in concert with CD28 co-stimulation (Waclavicek et al., 1997), CD47 positively regulates IL-2 production, thus supporting T cell proliferation (Ticchioni et al., 1997; Reinhold et al., 1997).

Together this evidence suggests that in healthy states SIRPα does not seem to play a role in the modulation of DC functions, and may enhance T cell stimulatory functions in presence of low antigen doses. However, in response to microbial challenge DC are primed for high level production of IL-12 p70, and SIRPα ligation can provide a negative feedback mechanism for prolonged IL-12 p70 production during DC:T cell interactions. In that the SIRPα$^+$ DCs expressed IL-12 p40 and IL-18, and are possibly 'primed' for activating cellular immune responses, the presence

of an inhibitory receptor, that modulates IL-12 expression could have an important role. The synergistic affect of IL-12 and IL-18 are in part mediated by IL-12 induction of IL-18 receptor (IL-18R) expression on T cells (Ahn et al., 1997). IL-12 and IL-18 have sequential affects of $CD4^+$ T cells; IL-12 is necessary for and precedes IL-18 action (Okamura et al., 1998). It seems that control of IL-12 production is necessary to prevent uncontrolled cell mediated responses and pathology, and SIRPα may provide an important mechanism for this *in vivo*.

5.3.8 Summary

Sheep afferent lymph contains $SIRP\alpha^+$ and $SIRP\alpha^-$ DCs. Both DC populations expressed MHC II, CD40, CD80, CD86, CD58 and CD11a that are typically expressed by DC in secondary lymphoid tissues. $SIRP\alpha^+$ and $SIRP\alpha^-$ DC differed in phenotype, the expression of individual markers and basal cytokine expression. $SIRP\alpha^+$ DCs appeared more homogenous than did $SIRP\alpha^-$ DCs. Moreover, $SIRP\alpha^+$ DCs phenotypically represented a more mature DC than $SIRP\alpha^-$ DCs. A major finding was that freshly isolated $SIRP\alpha^+$ DCs expressed mRNA transcripts for IL-12 p40 and IL-18, whereas these cytokines were undetectable in $SIRP\alpha^-$ DCs by PCR. Neither DC population appeared to express IL-6 or IL-10. These data suggest that ovine $SIRP\alpha^+$ DCs may be prone to inducing protent T cell reponses with a Th1 phenotype. $SIRP\alpha^-$ DCs, on the other hand, may be suppressive for T cell activation. This latter hypothesis is supported by the demonstration of apoptotic epithelia engulfments in $SIRP\alpha^-$ DCs in rat mesenteric lymph.

Further studies are needed to elucidate the biological roles of lymph DCs that selectivley express SIRPα. The current study indicates that DC populations defending epithelial tissues may have different biological roles *in vivo*.

Chapter 6: Final Discussion

It can be concluded that; 1) Two populations of DCs exist in sheep afferent lymph based on the presence or absence of SIRPα. 2) These DC populations differ in the expression of receptors involved in T cell stimulation and antigen detection and uptake. This may relate to cells being at different stages of maturation, or cells with distinct functions *in vivo*. 3) The SIRPα$^+$ and SIRPα$^-$ sheep lymph DCs displayed differential constitutive cytokine expression; the SIRPα$^+$ population expressed transcripts for IL-12 p40 and IL-18, whereas these were not detected in the SIRPα$^-$ DCs. These findings support the hypothesis that different DC populations preferentially bias T cell responses *in vivo* (Maldonado-Lopez et al., 1999; Hochrein et al., 2001).

SIRPα$^+$ DCs were essentially CD58$^+$, CD11a$^+$, WC6$^+$, CD45RA$^-$, expressed CD16, CD14, CD206, CD4 and CD8 at low levels or on minor sub-populations, and as a single population expressed higher levels of CD11c, CD40, CD80, CD86 and MHC II than did SIRPα$^-$ DCs. SIRPα$^-$ DCs were CD16$^-$, CD14$^-$, CD206$^-$, CD4$^-$, CD8$^-$, CD11a$^+$ and displayed heterogeneity of expression of CD58, CD11c, WC6 and CD45RA. The expression of MHC II, co-stimulatory molecules (CD40, CD80 and CD86) and adhesion molecules (CD58, CD11a) by SIRPα$^+$ DCs and significant proportions of SIRPα$^-$ DCs suggests that both populations possessed the capacity to interact with lymphocytes and participate in antigen presentation. These molecules may contribute to constitutive clustering of lymphocytes *in situ*. Additionally, the β2 integrins CD11a and CD11c, which are essential for leucocyte recruitment to tissues (Shuster et al., 1992) and homing to peripheral lymph nodes (Berlin-Rufenach et al., 1999), were expressed by both lymph DC populations and may be important for trafficking to the draining lymph nodes.

Expression of IL-12 p40 and IL-18 transcripts by SIRPα$^+$ DCs and not SIRPα$^-$ DCs may be related to phenotype characteristics. Expression of CD206, CD16 and CD14 by SIRPα$^+$ DCs may be an important factor. CD206 and CD16 have been demonstrated to act as signalling receptors triggering DC maturation and

IL-12 production (delaSalle et al., 1997; Shibata, Metzger and Myrvik, 1997). These receptors may interact with products of commensal bacteria of the skin, or circulating immune complexes. LPS, which uses CD14 as a binding receptor, enhances IL-18 expression in macrophages (Sections 3.2.6.1 and 4.4.7.1) and IL-12 p70 production in murine DCs (Reis e Sousa et al., 1997; Morelli et al., 2001). A component of gram negative bacterial cell walls, LPS is a potent stimulator of innate and inflammatory responses and instigates rapid innate immune responses (Ulevitch and Tobias, 1999). It is therefore unlikely that this pathogen-associated glycolipid plays a role in the constitutive expression of IL-12 p40 in this model where DCs were isolated from healthy sheep. This is not with counting however, that CD14, which signals through TLR4 and TLR2 may be involved in some other capacity. Data indicates that CD14 can also interact with numerous other peptides and lipids and is suggested to play a role in inflammatory responses to non-infectious injury (Pfeiffer et al., 2001). Thus, due to basal immunologic stimuli, or past infections, the expression of antigen receptors by the IL-12 p40, IL-18 expressing DC population ($SIRP\alpha^+$), implies a role in this function.

DCs provide important therapeutic targets (Vecchione et al., 2002). Understanding how the DC populations respond to *in vivo* activation signals, the capacity of DCs to present antigen to T cells, and the type of responses they induce is crucial for targeted manipulation *in vivo*, and the manipulation of DCs *in vitro* for *in vivo* adminsitration. Because of the high level of understanding of murine DC biology, it was useful to correlate ovine lymph DCs with murine DC populations. The phenotype and constitutive expression of IL-12 p40 and IL-18 suggests that $SIRP\alpha^+$ DCs may be related to the T-stimulatory $CD11c^+$ population described in murine secondary lymphoid organs (that inpart comprises the mature form of Langerhans' cells in the lymph nodes). CD45RA expression, a lack of 'myeloid' markers and no detectable IL-12 p40 or IL-18 transcripts suggests that sheep $SIRP\alpha^-$ DC may be comparable with murine $CD8\alpha^+$ / $B220^+$ pDCs. A number of *in vitro* investigations are imperative to test these hypotheses, such as the capacity for type I interferon production during exposure to virus, and the T cell-cytokine profiles induced in primed T cells following antigen-speicifc interactions with $SIRP\alpha^+$ and $SIRP\alpha^-$ DCs.

Different murine DC populations respond to pathogens and endogenous mediators differentially (Maldonado-Lopez et al., 1999). The lymphatic cannulation model could be utilised to investigate how lymph DCs respond to the activation of PAMP or endocytic receptors at the molecular level. SIRPα$^+$ DCs expressed CD16 (FcR) and CD206 (mannose receptor), and these receptors could be ligated and activated with anti-CD16 and anti-CD206 -coated polystyrene beads. Levels of cytokine mRNA could be analysed using the RPA (following collection of lymph DCs and cryopreservation; or pooling DC RNA over time). Furthermore, in the interests of DC biology, it would be interesting to investigate the role of SIRPα and CD45RA on the DC populations. That the lymph DC populations differentially express these molecules may indicate a role in regulating DC responses and cytokine production. Cytokine responses could be analysed using the RPA (following the considerations mentioned above) following activation of DCs by antigen or microbial products, and the subsequent ligation and cross-linking of SIRPα and CD45RA. In addition, the uptake of apoptotic cells has major implications for antigen-specific tolerance. Differential expression of CD11c on the DC populations indicates a difference for a capacity for apoptotic cell uptake, and cross-presentation of endogenous antigen to CD8 T cells. Whether ovine lymph DCs are capable of inducing antigen specific tolerance is not known, and could be investigated by a series of *in vitro* experiments using primed T cells as targets (Martin et al., 2002).

The development of a RNase protection assay for nine sheep cytokines, including IL-18 provided a valuble technique for assessing cytokine mRNA levels concordantly. This system has been utilised for analysing the responses of antigen-specific lymphocyte populations *in vivo* (Gossner et al., 2002), and provides a valuable tool for quantifying levels of cytokine expression in sheep. Ability to measure IL-18 expression has important implications for innate immune responses, and enhancing IL-18 during vaccination trials enhances DC immunogenicity (Tastsumi et al., 2002; Goto et al., 2002). The capacity to measure IL-18 levels in ruminants could promote work to enhance immunogenicy to antigens, and be important for vaccination trials.

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Zhang Hoover, J., Sutton, A., and Stein Streilein, J. (2001). CD40/CD40 ligand interactions are critical for elicitation of autoimmune-mediated fibrosis in the lung. *Journal of Immunology* **166,** 3556.

Zhu, C., Gagnidze, K., Gemberling, J. H., and Plevy, S. E. (2001). Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. *Journal of Biological Chemistry* **276,** 18519.

Appendix 1: General Solutions

Unless otherwise stated, all chemicals were obtained from Sigma or Merck. All solutions were prepared in sterile distilled H_2O.

Hepes buffered saline (HBS)
0.8% w/v $NaCl_2$
10 mM HEPES
pH 7.4

Hank's buffered salt solution (HBSS)
150 mM NaCl
25 mM HEPES
5 mM KCl
5 mM glucose
1 mM $CaCl_2$
1 mM KH_2PO_4
I mM $MgSO_4$
0.001% phenol red
pH 7.0

Luria-Bertani (LB) broth
1%w/v tryptone
0.5%w/v yeast extract
0.17M NaCl
pH 7.0

LB Agar
LB containing 1.5% Bacto-Agar (Oxoid)
1 mM EDTA

Phosphate buffered saline (PBS)
137 mM NaCl
27 mM KCl
8 mM Na_2HPO_4
15 mM KH_2PO_4
pH 7.2

SDS sample buffer
2% SDS
20% glycerol
0.125 M Tris pH 6.8
0.004% bromoblue
5% mercaptoethanol
pH 7.4

SOC medium
LB containing 20 mM glucose
20 mM $MgCl_2$

SOB medium
LB
80 mM $MgCl_2$

TE buffer
10 mM Tris-HCl (pH8.0)
1 mM EDTA

TAE buffer
40 mM Tris-acetate

TB buffer
10 mM PIPES
55 mM $MnCl_2$
15 mM $CaCl_2$
250 mM KCl

Appendix 2: Commercial Suppliers

Agilent Technologies (UK) Ltd., Lakeside, Cheadle Royal Business Park, Stockport, Cheshire, SK8 3GR.
www.agilent.com

Amersham Pharmacia Biotech (UK) Ltd., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA
www.apbiotech.com

Axis-Shield PoC AS, P.O. Box 6863 Rodelokka, Oslo, Norway N-0504
www.axis-shield-poc.com

Beckman Coulter (UK) Ltd., Oakley Court, Kingsmead Business Park, London Road, High Wycome, Buckinghamshire, HP11 1JU
www.beckman.com

Becton Dickinson (UK) Ltd., Between Towns Road, Cowley, Oxford, Oxfordshire, OX4 3LY
www.bd.com

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD
www.bio-rad.com

Biowhittaker (UK) Ltd., 1 Ashville Way, Wokingham, Berkshire, RG41 2PL

European collection of cell cultures (ECACC), CAMR, Porton Down, Salisbury, Wiltshire, SP4 OJG
www.ecacc.org.uk

Fisher Scientific (UK) Ltd., Bishop Meadow Road, Loughborough, Leicesterhire, LE11 5RG
www.fisher.co.uk

GraphPad Software, San Diego, California, USA
www.graphpad.com

Invitrogen Life Technologies, Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF
www.flowgen.co.uk

Merck Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leics, LE17 4XN
www.merckeurolab.ltd.uk

Appendix 2 Commercial suppliers

Millipore (U.K.) Limited, Units 3&5 The Courtyards, Hatters Lance, Watford WD18 8YH
www.millipore.com

Miltenyl Biotec Ltd. Almac House, Church Lance, Bisley, Surrey, GU24 9DR
www.miltenylbiotech.com

MWG Biotech AG, Mill Court, Featherstone Road, Wolverton Mill South, Milton Keynes, NK12 5RD
www.mwg-biotech.com

Nalgene Nunc International, 75 Panorama Creek Drive, P.O. Box 20365, Rochester, NY 14602-0365
www.nalgenenunc.com

National Diagnostics, Unit 4, Fleet Business Park, Itlings Lane, Hessle, Hull HU13 9LX
www.nationaldiagnostics.com

New Englands Biolabs (UK) Ltd., 73 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY
www.neb.com

Philips electronics UK. Ltd., The Philips Centre, 420-430 London Road, Croydon, Surrey, CR9 3QR.
www.philips.com

Promega UK Ltd., Delta House, Chilworth Research Centre, Southampton, SO16 7NS
www.promega.com

Roche Diagnostics Ltd. Bell Lane, Lewes, East Sussex, BN7 1LG
http://biochem.roche.com

QIAGEN Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 2AX
www.qiagen.com

Sigma-Aldrich Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT
www.sigma-aldrich.com

ThermoHybaid, Action Court, Ashford Road, Ashford, Middlesex, TW15 1XB
www.thermohybaid.com

Table 1 Specificity and source of monoclonal antibodies (mAb). The panel of mAb using in flow cytometry and MACS experiments is shown. mAb isotype, antigen specificity and original references are listed. The mAb preparation is indicated in green; saturated hybridoma supernatant, S/S; ascitic fluid, A/F, and the dilution used for staining of cells for flow cytometry and / or MACS is shown in blue; 1:1 indicates that the mAb preparations were used un-diluted. Some mAb recognise antigen that have no established CD homologue but have been given workshop cluster (WC) numbers at the International Ruminant Leukocyte Antigen Workshops (1991, 1993) (Parsons et al., 1993,; Howard et al., 1991). SIRPα is the ruminant homologue of human signal inhibitory receptor protein (Brooke et al.,1998). m, murine; r, rat.

APPENDIX 3: MONOCLONAL ANTIBODIES

mAb Code	Isotype[a]	Antigen	Reference
VPM5 A/F 1:100	mIgM	**ovCD1b**	Budjoso et al. (1989)
36F A/F 1:150	$mIgG_{2a}$	**ovCD2**	Mackay et al. (1988)
ST4 S/S 1:1	$mIgG_{2a}$	**ovCD4**	Maddox et al. (1985)
SBU-T8 S/S 1:1	$mIgG_{2a}$	**ovCD8**	Maddox et al. (1985)
F10-150 S/S 1:1	$mIgG_1$	**ovCD11a**	Gupta et al. (1993)
ILA-15 S/S 1:1	$mIgG_1$	**ovCD11b**	Gupta et al. (1993)
OM1 S/S 1:1	$mIgG_1$	**ovCD11c**	Gupta et al. (1993)
VPM67 A/F 1:1000	$mIgG_1$	**ovCD14**	Hopkins and Gupta (1996)
VPM64 S/S 1:1	$mIgG_1$	**ovCD23**	Hopkins and Gupta (1996)
VPM63 S/S 1:1	$mIgG_1$	**ovCD32**	Hopkins and Gupta (1996)
ILA-156 A/F 1:50	mIgG	**ovCD40**	J. Naessens (personal communication)
73B S/S 1:1	$mIgG_1$	**ovCD45RA**	Dutia et al. (1993)
T11TS S/S 1:1	$mIgG_1$	**ovCD58**	Hunig et al. (1985)
ILA-159 A/F 1:1000	mIgG	**ovCD80**	J. Naessens (personal communication)
ILA-190 A/F 1:1000	mIgG	**ovCD86**	J. Naessens (personal communication)
3.29B1 S/S 1:1	$mIgG_1$	**bovCD206**	Howard et al. (1997)
SW73.2 A/F 1:2000	$rIgG_{2a}$	**ovMHC II β chain**	Hopkins et al. (1986)
VPM54 S/S 1:1	$mIgG_1$	**ovMHC II**	Dutia et al. (1995)
IAH-CC15 A/F 1:200	$mIgG_{2a}$	**ovWC1**	Clevers et al. (1990)
ILA-53 A/F 1:100	$mIgG_{2a}$	**ovWC6**	Dutia et al. (1993)
ILA-24 S/S 1:1	$mIgG_1$	**bovSIRPα**	Ellis et al. (1988); Brooke et al. (1998)
VPM30 A/F 1:500	mIgM	**ovPan B cell**	Bird et al. (1995)

APPENDIX 4 OLIGONUCLEOTIDE PRIMER SEQUENCES

Primer designation	Sequence 5'-3'	Specificity
G1899241F Sense	ATGGCTGCTGAACC	Human IL-18 cDNA. 178-192 nt.
G1899241R Antisense	GGTCTCTCTCTTTTTCACAAG	Human IL-18 cDNA. 681-701 nt.
AB01003F Sense	CGGAAGACAATTGCATCAGC	Porcine IL-18 cDNA. 14-33 nt.
AB01003R Antisense	CAGTGAACATTATAG	Porcine IL-18 cDNA. 547-561 nt.
Y11133F Sense	TAGAAGACAATTGCATCAAC	Canine IL-18 cDNA 17-36 nt.
ConIL-18R Antisense	GATTTATCYCCAYWTTCATCC	Consensus IL-18 531-551 nt.
AF124789F Sense	ATGGCTGCAGAACAAGTAGA	Bovine IL-18 cDNA 150-169
AF124789R Antisense	CTAGTTCTTGTTTTGAACAGTGAACAT	Bovine IL-18 cDNA 705-731
RACE1 Antisense	GTCCTGGAACACTTCTCTGAAAG	Ovine IL-18 cDNA 411-434 nt.
RACE2 Antisense	CACTGCACAGAGATGGTTACAG	Ovine IL-18 cDNA 288-310 nt.
RACE3 Antisense	GGTCTGGGGTGCATTATCTGAAC	Ovine IL-18 cDNA 219-242 nt.
RACE4 Sense	GGAATCAGATCACTTTGGCAAGC	Ovine IL-18 cDNA 98-121 nt.
RACE5 Sense	GGAATCAGATCACTTTGGCAAGC	Ovine IL-18 cDNA 223-243 nt.
Oligo (dT)-anchor	GACCACGCGTATCGATGTCGACT(16)V	
anchor	GACCACGCGTATCGATGTCGAC	
X02813F1 Sense	CAAGACCAGGAGGAATTCCG	Ovine ATPase cDNA 3069 - 3088
X02813R1 Antisense	GGGCAGTAGGAAAGGAAAGC	Ovine ATPase cDNA 3154 - 3173
X02813R2 Antisense	CACCCATTCCAGGGCAGTAGG	Ovine ATPase cDNA 3164 - 3184
AF030943R1 Antisense	AGGCCATGTGGACCATGAGG	Ovine GAPDH cDNA 1kb insert
AF030943F2 Sense	CACTACCATGGAGAAGGCTGG	Ovine GAPDH cDNA 171-191 nt.
AF030943R2 Antisense	GTGGTTCACGCCCATCACA	Ovine GAPDH cDNA 257-275 nt.
M96845F Sense	GCGCTGGTCTGCTTACTGGTATG	Ovine IL-4 cDNA 86 – 108 nt.

Appendix 4 Primer Sequences

Primer designation	**Sequence 5'-3'**	**Specificity**
M96845R Antisense	GATACGCCTAAGCTCAATTCC	Ovine IL-4 cDNA 275 – 295 nt.
X68723F Sense	ACTCCCTCTTCACAAGCGC	Ovine IL-6 cDNA 23-41 nt.
X68723R Antisense	ACACTCGTCATTCTTCTCACATAT	Ovine IL-6 cDNA 229-252 nt.
S74436F Sense	AACGAGAGCCAGAAGAAACC	Ovine IL-8 cDNA 15-34 nt.
S74436R Antisense	TCATGGATCTTGCTTCTCAG	Ovine IL-8 cDNA 343-362 nt.
Z29362F Sense	TACCCACTTCCCAGCCAG	Ovine IL-10 cDNA 161-179 nt.
Z29362R Antisense	CGTTGTCATGTAGGATTCTATGTAG	Ovine IL-10 cDNA 569-593 nt.
AF004024F1 Sense	GTACACCTGTCACAAAGGAGGCG	Ovine IL-12 p40 cDNA 261-281 nt.
AF004024F2 Sense	ATGCTGGGCAGTACACCTG	Ovine IL-12 p40 cDNA 251-270 nt.
AF004024R Antisense	CCTCCTGACACTCCACTGTG	Ovine IL-12 p40 cDNA 570-589 nt.
AJ401033F Sense	GACAATTGCATCAGCTTTGTGG	Ovine IL-18 cDNA 22 – 42 nt.
AJ401033R Antisense	CCTGGCTAATGAAGAGAACTTG	Ovine IL-18 cDNA 342 – 362 nt.
X54796F Sense	GATGAAGAGCTGCACCCAACA	Ovine IL-1β cDNA 122-143 nt.
X54796R Antisense	ATGGCCACGATGACCGA	Ovine IL-1β cDNA 228-244 nt.
X53561F Sense	GTCCTCAAGAGGATG	Ovine GM-CSF cDNA 2 – 26 nt.
X53561R Antisense	GTCAAGGAGCCCGTG	Ovine GM-CSF cDNA 283 – 297 nt.
X52640F Sense	CTCTCTCCTAAACGATGAAATACAC	Ovine IFNγ cDNA 42 – 66 nt.
X52640R Antisense	AAGTAGAAGGAGACAATTTGGCT	Ovine IFNγ cDNA 263 – 285 nt.
X76916F Sense	TGGACACCAACTACTGCTTCAG	Ovine TGFβ cDNA 839 - 860
X76916R Antisense	CGATCATGTTGGACAACTGCTC	Ovine TGFβ cDNA 1138 - 1150
X76916F Sense	TGGACACCAACTACTGCTTCA	Ovine TNFβ cDNA 839 – 859 nt.
X76916R Antisense	CGATCATGTTGGACAACTGCTC	Ovine TNFβ cDNA 1138 – 1150 nt.
X55152F Sense	ATGAGCACCAAAAGCATGATCCG	Ovine TNFα cDNA 159-182 nt.
X55152R Antisense	ACCCAGGAGCAACCAGAAGAG	Ovine TNFα cDNA 260-280 nt.

Appendix 4 Primer Sequences

Primer designation	**Sequence 5'-3'**	**Specificity**
pGEM-XF Sense	CCTAGGCATCCGCGGGAATTCGA BamH I	pGEM-T-easy vector
pGEM-XF Antisense	CACTAGTGAATTCGCGGCATGCTGCAG Sph I	

Y = C or T; W = A or T; V = A, C or G

APPENDIX 5: CYTOKINE CDNA CLONES

Clone	Identification	Backbone	Source
ovIL-1β	X54796	pBluescript II	Dr. Carolyn Fiskerstrand (Vet. Pathology, University of Edinburgh)
ovIL-4	M96845	pUC119	Dr. Colin McInness (Moredun Institute, Edinburgh)
ovIL-6	X68723	pTZ18	Dr. Bahram Ebrahim (Vet. Pathology, University of Edinburgh)
ovIL-8	S74436	pBluescript II	Dr. Bahram Ebrahim (Vet. Pathology, University of Edinburgh)
ovIL-10	Z29362	pCRII	Dr. Bahram Ebrahim (Vet. Pathology, University of Edinburgh)
ovIL-12 p40	AF004024	pCRII	Dr. Bahram Ebrahim (Vet. Pathology, University of Edinburgh)
ovIL-18	AJ401033	pGEM-T-easy	Produced during this thesis.
ovGM-CSF	X53561	pBluescribe	Dr. Colin McInness (Moredun Institute, Edinburgh)
ovIFNγ	X52640	pBluescribe	Dr. Colin McInness (Moredun Institute, Edinburgh)
ovTGFβ	X76916	pGEM-T-easy	Dr. Chris Woodall (Caledonian University, U.K.)
ovTNFα	X55152	pBluescript II	Dr. Bahram Ebrahim (Vet. Pathology, University of Edinburgh)
ovATPase	X02813	pBluescript II	Dr. Bahram Ebrahim (Vet. Pathology, University of Edinburgh)
ovGAPDH	AF030943	pBluescript II	Dr. Bahram Ebrahim (Vet. Pathology, University of Edinburgh)

APPENDIX 6: RIBOPROBE TEMPLATE SEQUENCES

Sequences of the cDNA clone templates for riboprobe synthesis in the sheep cytokine RNase protection assay are presented below. Regions of the vector pGEM®-T-easy are shown in red, and pGEM®-11-Zf in blue.

IL-1β

AGTCGGCCGAGCTCGAATTCGTCGACCTCGAGGGATCCGCGGGAATTCGATTGATGAAGA
GCTGCACCCAACACCTGGACCTCGGCTCCATGGGAGATGGAAACATCCAGCTGCAGATTT
CTCACCAGCTCTACAACAAAAGCTTCAGGCAGGTAGTGTCGGTCATCGTGGCCATAATCAC
TAGTGAATTCGCGGCATGCA

IL-4

GGGCGAATTGGCCAAGTCGGCCGAGCTCGAATTCGTCGACCTCGAGGGATCCGCGGGAAT
TCGATTTTGCCTCACACTGTCAGTGCAAATAGAGATACTATTAATGGGTCTCACCTCCCA
GCTGATCCCAGCGCTGGTCTGCTTACTGGTATGTACCAGCCACTTCGTCCATGGACACAT
GTGTGATATTACCTTAGAAGAGATCATCAAAACGCTGAACATCCTCACATCGAGAAAGAA
TTCATGCATGGAGCTGCCTGTAGCAGAGTCTTTGCAATCACTAGTGAATTCGCGGCATGC

IL-6

CCAAGTCGGCCGAGCTCGAATTCGTCGACCTCGAGGGATCCGCGGGAATTCGATTAGATC
CTGAAGGAAAAGATCGCAGGTCTAATAACCACTCCAGCCACACACACTGACATGCTGGAG
AAGATGCAGTCCTCAAACGAGTGGGTAAAGAACGCAAAGGTTATCATCATCCTGAGAAGC
CTTGAGAATTTCCTGCAGTTCAGCCTGAGAGCTATTCGGATGAAGTAGAATCACTAGTGA
ATTCGCGGCATGCATAAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGG

IL-8

GGGAATTCGATTAACGAGAGCCAGAAGAAACCTGACAAAAAGCCTCTTGCTCAAGTATGA
CTTCCAAGCTGGCTGTTGCTCTCTTGGCCGCTTTCCTGCTCTCTGCAGCTCTGTGTGAAG
CTGCAGTTCTGTCAAGAATGAGTACAGAACTTCGATGCCAATGCATAAAAACACATTCCA
CACCTTTCCACCCCAAATTTATCAAAGAACTGAGAGTTATTGAGAGTGGGCCACACTGCG
AAAATTCAGAAATCATTGTTAAGCTTACCAACGGAAAAGAGGTGTGCTTAGACCCCAAGG
AAAAGTGGGTGCAGAAGGTTGTGCAGGCATTTTTGAAGAGAGCTGAGAAGCAAGATCCAT
GAAATCACTAGTGAATTCGCGGCATGCATAAGCTTGAGTATTCTATAGTGTCACCTAAAT
AGCTTGGCGT

IL-10

AATTGGCCAAGTCGGCCGAGCTCGAATTCGTCGACCTCGAGGGATCCGCGGGAATTCGATT
ACAAAGCCATGAGTGAGTTTGACATCTTCATCAACTACATAGAATCCTACATGACAACGAA
GATGTAAAACTGAAGCATTCTAGGGAAGGAGACCTCCAGCATGGTGACTCGACTAGACTCC
CCGACATAAACCTCTGAAATCCGACCCAGGGTTCTGGGAGAGCAGAGCCAGCTCCCTGGAG
ACCTCCACTGTGCCTCTCCCCTAGAGTATTTATTACCTCTGATACCTCAGCTCCCACATCT
ATTTATTTACTGAGCTTCTCTGTGAACAATCACTAGTGAATTCGCGGCATGCA

IL-12 p40

TCGAATTCGTCGACCTCGAGGGATCCGCGGGAATTCGATGTACACCTGTCACAAAGGAGG
CGAGGTTCTGAGTCGTTCACTCCTCCTGCTGCACAAAAAGGAAGATGGAATTTGGTCCAC
TGATATTTTAAAGGATCAGAAGAACCCAAAGCTAAGAGTTTTTTAAAATGTGAGGCAAAG
GATTATTCTGGACACTTCACCTGCTCGTGGCTGACAGCAATCAGTACTAATCTGAAATTC
AGTGTCAAAAGCAGCAGAGGCTCCTCTGACCCCCGAGGGGTGACGTGCGGAGCAGCGTCC
CTCTCAGCAGAGAAGGTCAGCATGGACCACAGGGAGTATAACAAGTACACAGTGGAGTGT
CAGGAGGGAATCACTAGTGAATTCGCGGCA

Appendix 6 Riboprobe Temple Sequences

IL-18

TTGACAATTGCATCAGCTTTGTGGAAATGAAATTTATTAACAATACACTTTATTTTGTAG
CTGAAAATGGCGAAGACCTGGAATCAGATCACTTTGGCAAGCTTGAACCTAAGCTCTCAA
TCATACGAAATTTGAACGACCAAGTTCTCTTCATTAGCCAGGAATCACTAGGGAATTCGC
GGCATGCATAAGCTTGAGTATTCTATAGTGTCACCTAAAT

GM-CSF

TGGCCAAGTCGGCCGAGCTCGAATTCGTCGACCTCGAGGGATCCCGGGAATTCGATTGTCC
TCAAGAGGATGTGGCTGCAGAACCTGCTTCTCCTGGGCACTGTGGTCTGCAGCTTCTCCGC
ACCCACTCGCCAACCCAGCCCTGTCACCCGGCCCTGGCAGCATGTGGATGCCATCAAGGAG
GCCCTGAGCCTTCTGAACGACAGCACTGACACTGCTGCTGTGATGGATGAAACAGTAGAAG
TCGTCTCTGAAATGTTTGACTCCCAGGAGCCGACATGCCTGCAGACTCGCCTGGAGCTGTA
CAAGCAGGGCTGCGGGGCAGCCTCACCAGTCTCACGGGCTCCTTGACAATCACTAGTGAAT
TCGCGGCATGCA

IFNγ

GGGAATTCGATTCTCTCTCCTAAACGATGAAAGACACAAGCTCCTTCTTAGCTTTACTGC
TCTGTGTGCTTTTGGGTTTTTCTGGTTCTTATGGCCAGGGCCCATTTTTTAAAGAAATAG
AAAACTTAAAGGAGTATTTTAATGCAAGTAGCCCAGATGTAGCTAAGGGTGGGCCTCTTT
TCTCAGAAATTTTGAAGAATTGGAAAGAGGAGAGTGACAAAAAGATTATTCAGAGCCAAA
TTTTCTCCTTCTACTTAATCACTAGTGAATTCGCGGCATGCATAAGCTTGAGTATTCTAT
AGTGTCACCTAAATA

TGFβ

TGGCCAAGTCGGCCGAGCTCGAATTCGTCGACCTCGAGGGATCCGCGGGAATTCGATCCG
AGCCCTGGACACCAACTACTGCTTCAGCTCCACAGAAAAGAACTGCTGTGTTCGTCAGCT
CTACATTGACTTCCGGAAGGACCTGGGCTGGAAGTGGATTCACGAACCCAAGGGCTACCA
CGCCAATTTCTGCCTGGGGCCCTGTCCCTACATCTGGAGCCTGGACACACAGTACAGCAA
GGTCCTGGCCCTGTACAACCAGCACAACCCGGGCGCATCGGCGGCGCCGTGCTGCGTGCC
TCAGGCGCTGGAACCCCTGCCCATCGTGTACTACGTGGGCCGCAAGCCCAAGGTGGAGCA
GTTGTCCAACATGATCGAATCACTAGTGAATTCGCGGCATGCATAAG

TNFα

TATAGGGCGAATTGGCCAAGTCGGCCGAGCTCGAATTCGTCGACCTCGAGGGATCCGCCC
ATGAGCACCAAAAGCATGATCCGGGATGTGGAGCTGGCGGAGGAGGTGCTCTCCAACAAA
GCAGGGGGCCCCCAGGGCTCCAGAAGTTGCTGGTGCCTCAGCCTCTTCTCCTTCCTCCTG
GTTGCAGGAGCCACCACGCTCTTCTGGGGCTAGAGCGGCCGCATGCATAAGCTTGAGTAT

GAPDH

GGGAATTCGATTGGAAGCTGTGGCGTGATGGCCGAGGGGCTGCCCAGAACATCATCCCTG
CTTCTACTGGCGCTGCCAAGGCCGTGGGCAAGGTCATCCCTGAGCTCAACAATCACTAGT
GAATTCGCGGCATGCATAAGCTTGAGTATTCTATAGTGTCACCTAAATA

Appendix 7(a). Ratio of cytokine mRNA levels in adherent bronchoalveolar cells (non-purified); LPS stimulated versus medium controls.

The RPA was used to evaluate cytokine mRNA abundance in sheep adherent bronchoalveolar cells cultured with or without 1 μg/ml LPS. The levels of individual transcripts were normalised relative to GAPDH expression, and expressed as an arbitrary unit. The ratio of expression of each cytokine in LPS stimulated cultures is plotted relative to medium controls on a $\log_{10}$ scale, individual samples are plotted as a single point. Where no transcripts were detected in either the control or LPS stimulated cells, a value of zero is given, therefore comparisons of the $\log_{10}$ value of these data cannot be made, and are not included in these graphs. Fold differences of cytokine expression between experimental groups are indicated on the graphs. These experiments involved cells isolated from three sheep. The figures are as follows:

1a IL-12 p40: 1, 4, 6 hours

1b IL-12 p40: 16, 20 hours

2 IL-10: 4, 6, 16 hours

3 IL-6: 4, 6, 16 hours

4a GM-CSF: 1, 4 hours

4b GM-CSF: 6, 16 hours

5a IL-8: 1, 4, 6 hours

5b IL-8: 16, 20 hours

6a IL-1β: 1, 4, 6 hours

6b IL-1β: 16, 20 hours

7 TNFα: 1 hour

8a TGFβ: 1, 4 hours

8b TGFβ: 6, 16 hours

9a IL-4: 1, 4, 6 hours

9b IL-4: 16, 20 hours

10a IFNγ: 1, 4, 6 hours

10b IFNγ: 16, 20 hours

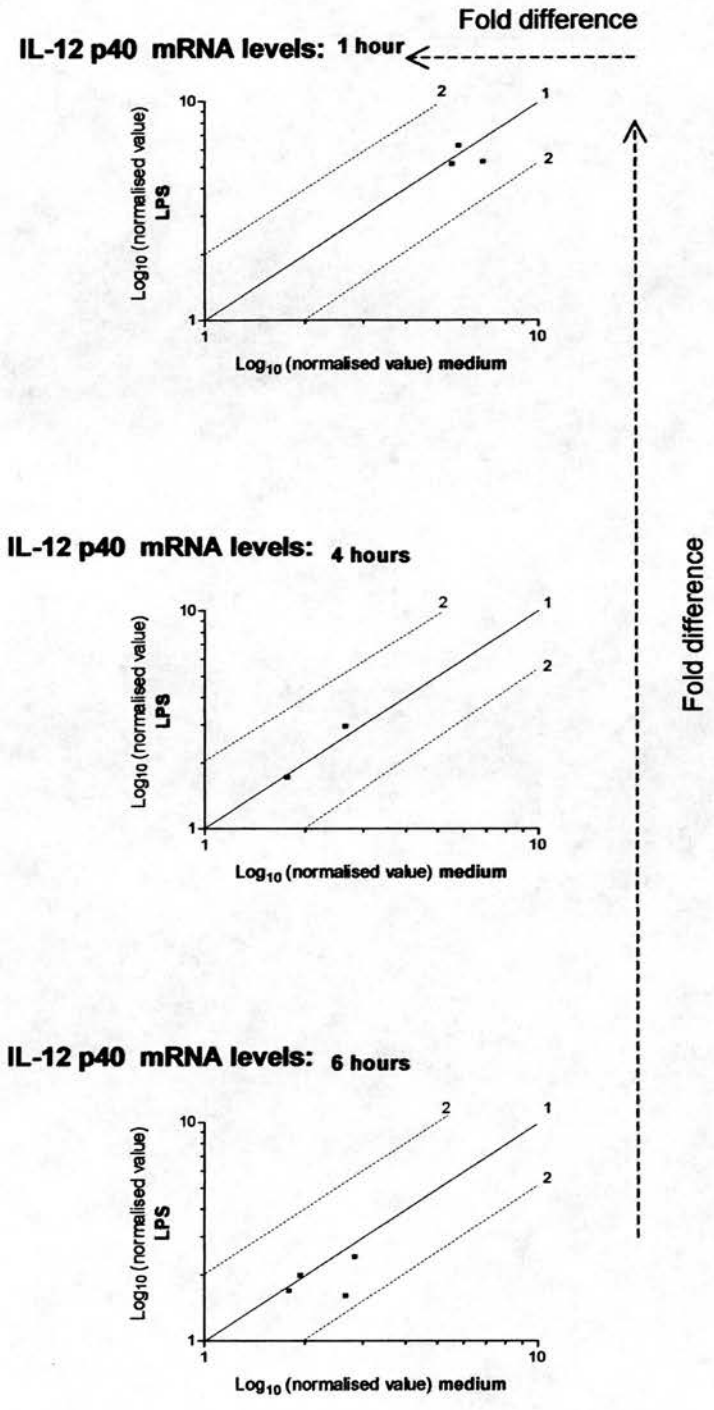

Figure 1a. Expression of IL-12 p40.

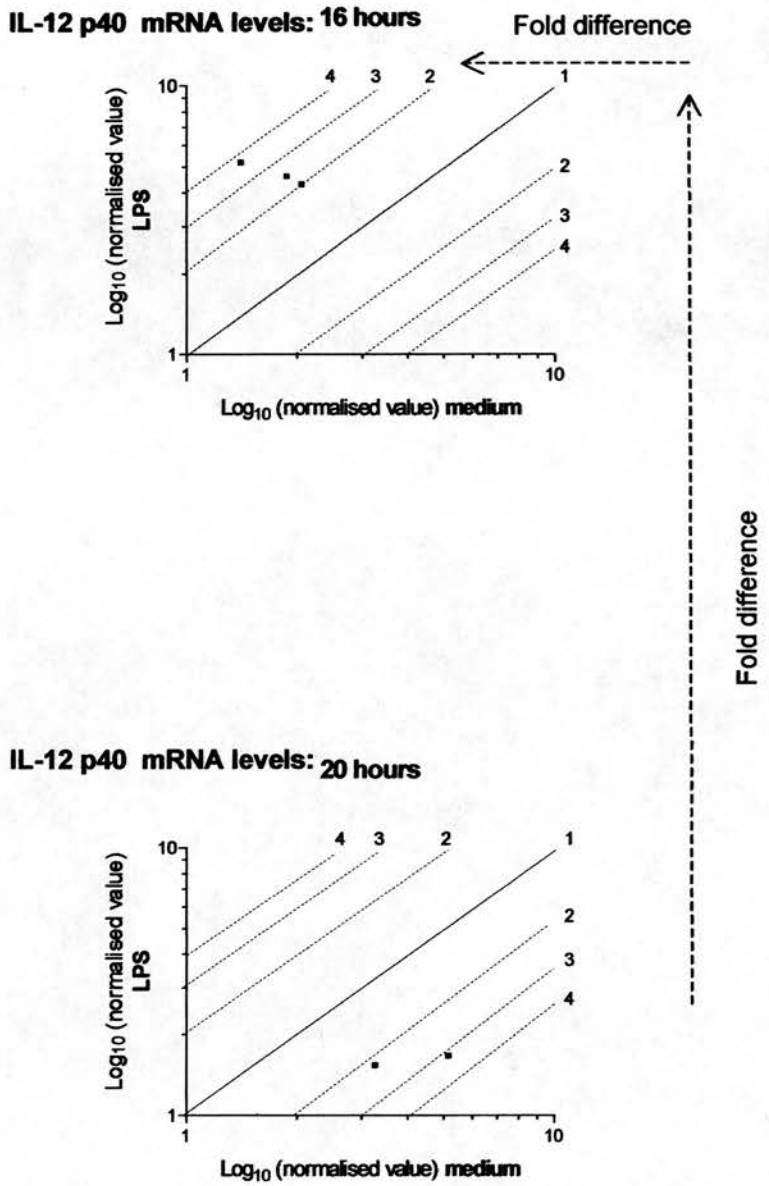

Figure 1b. Expression of IL-12 p40.

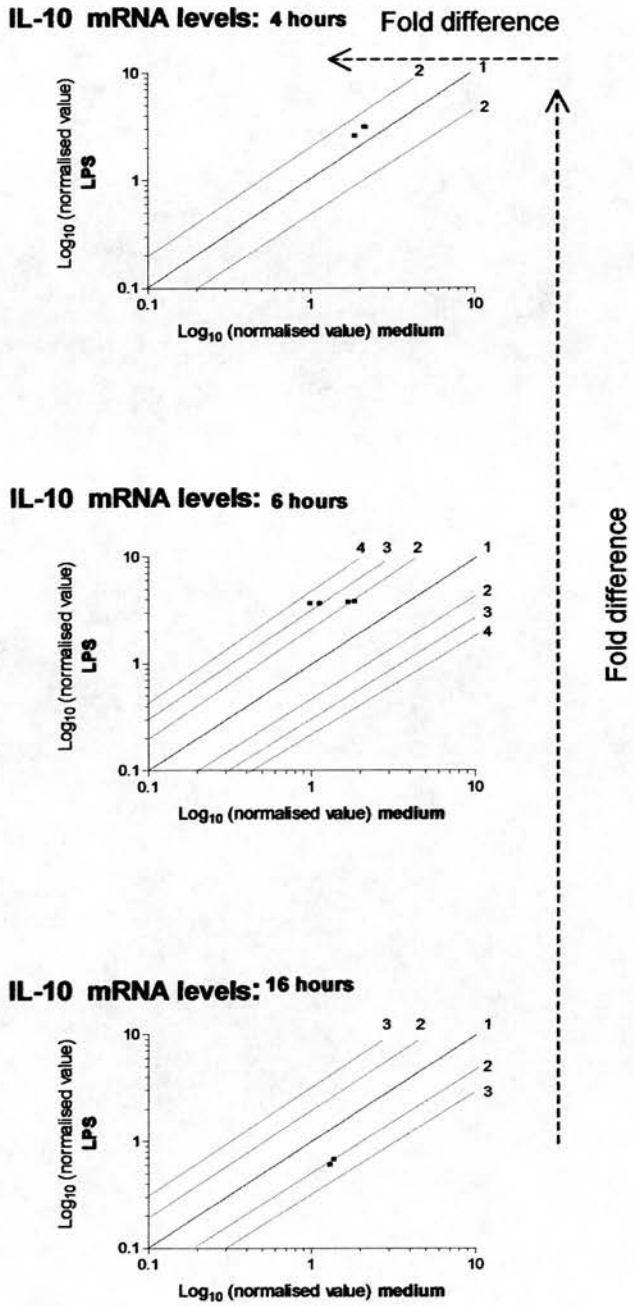

Figure 2. Expression of IL-10.

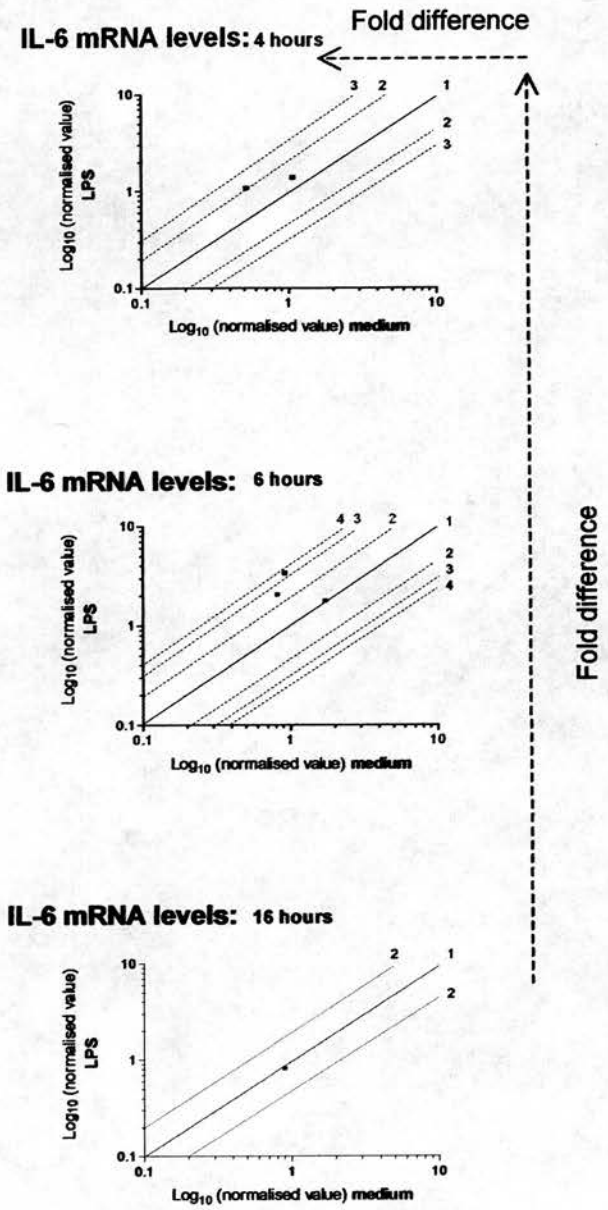

Figure 3. Expression of IL-6.

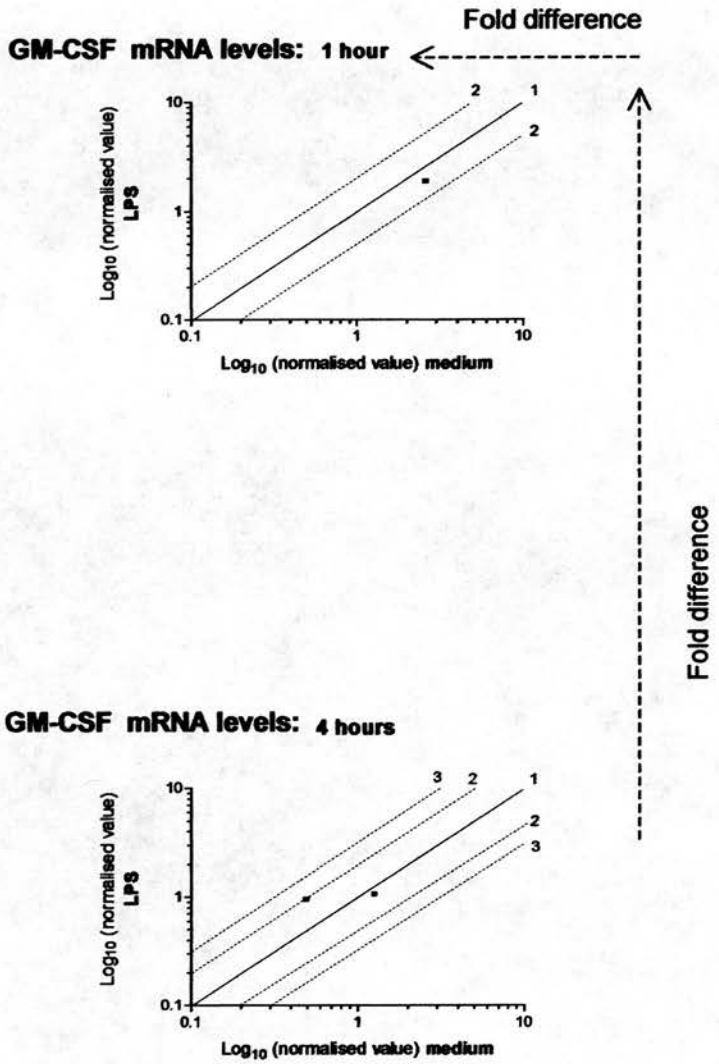

Figure 4a. Expression of GM-CSF.

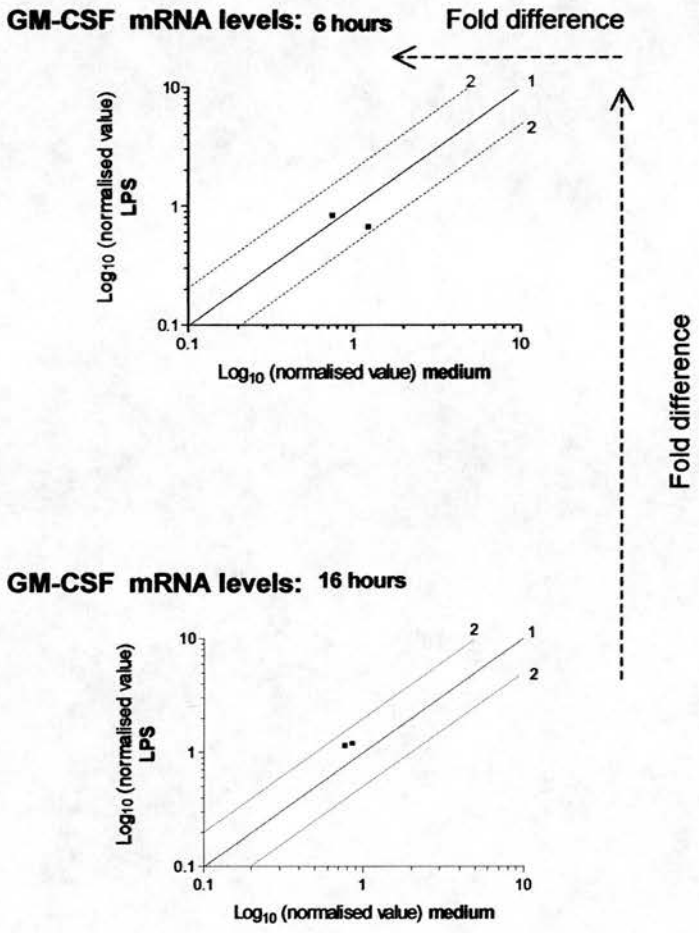

Figure 4b. Expression of GM-CSF.

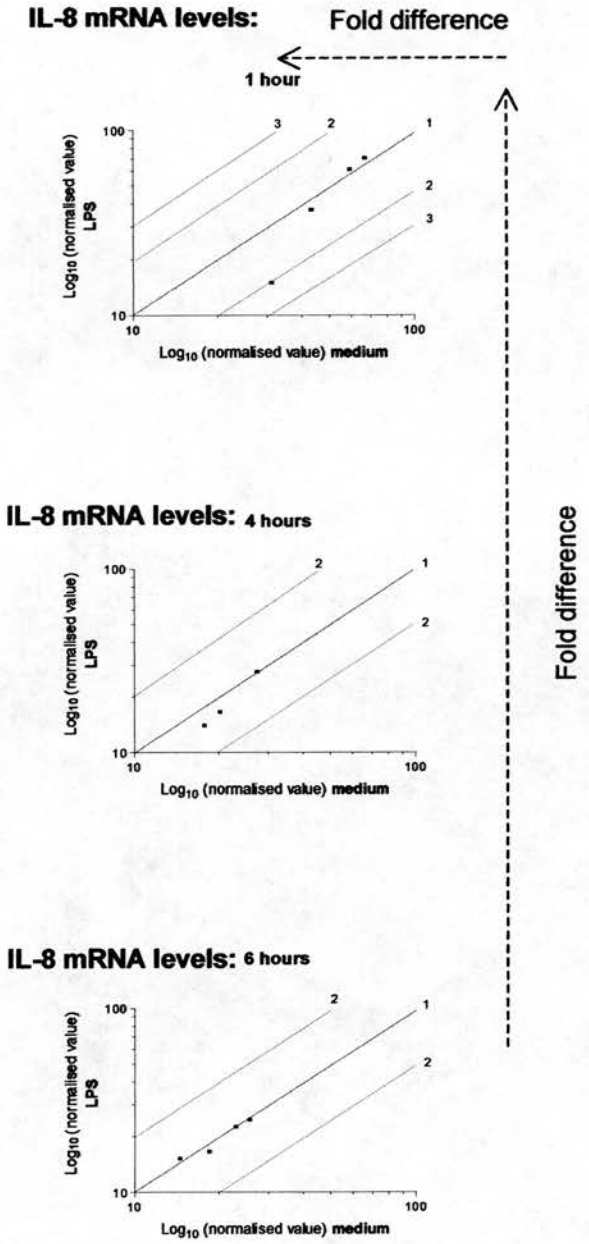

Figure 5a. Expression of IL-8.

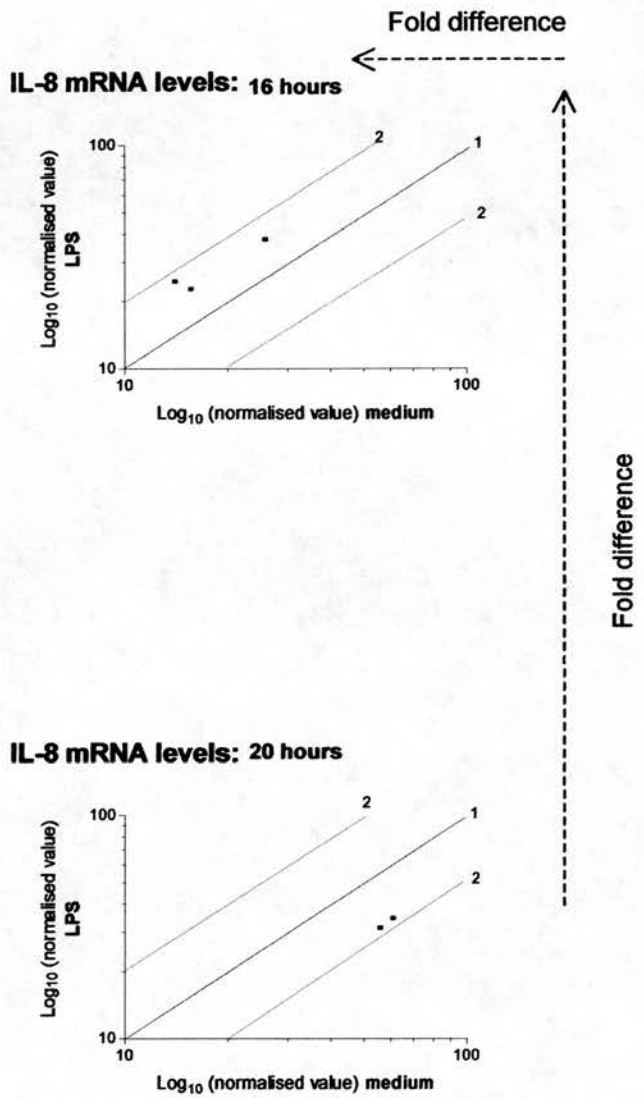

Figure 5b. Expression of IL-8.

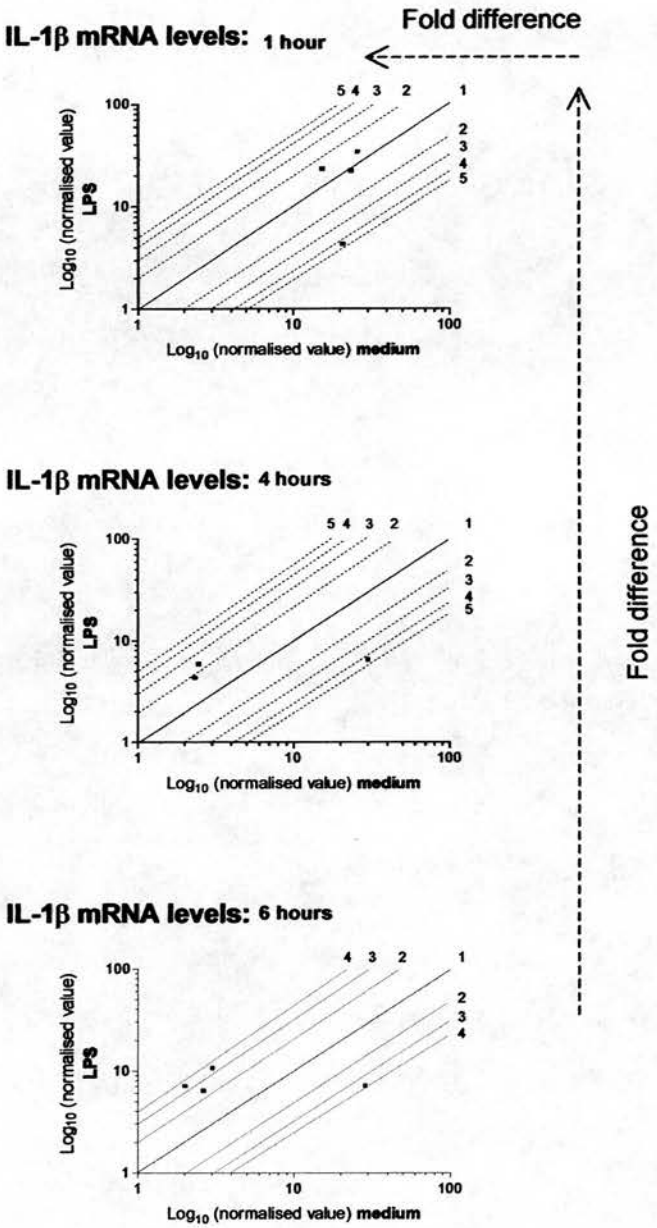

Figure 6a Expression of IL-1β.

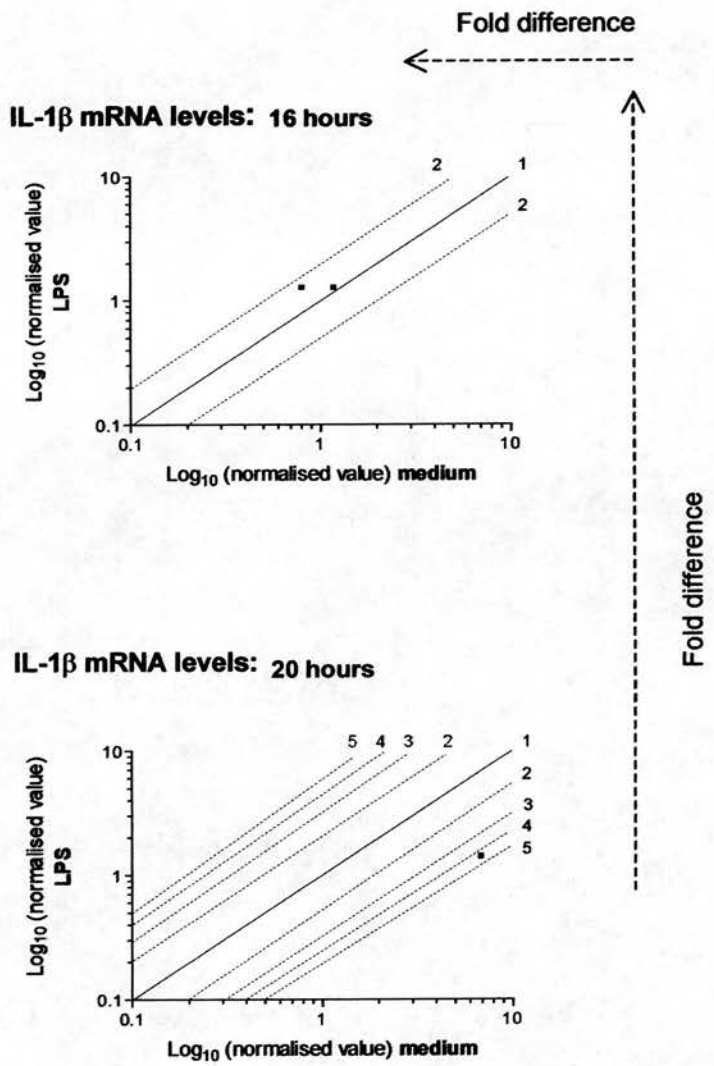

Figure 6b Expression of IL-1β.

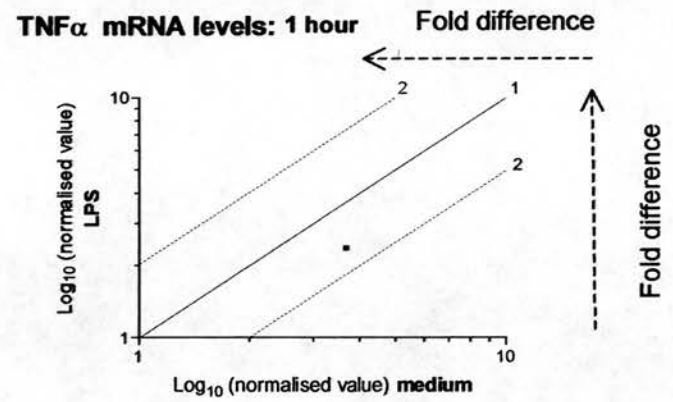

Figure 7. Expression of TNFα.

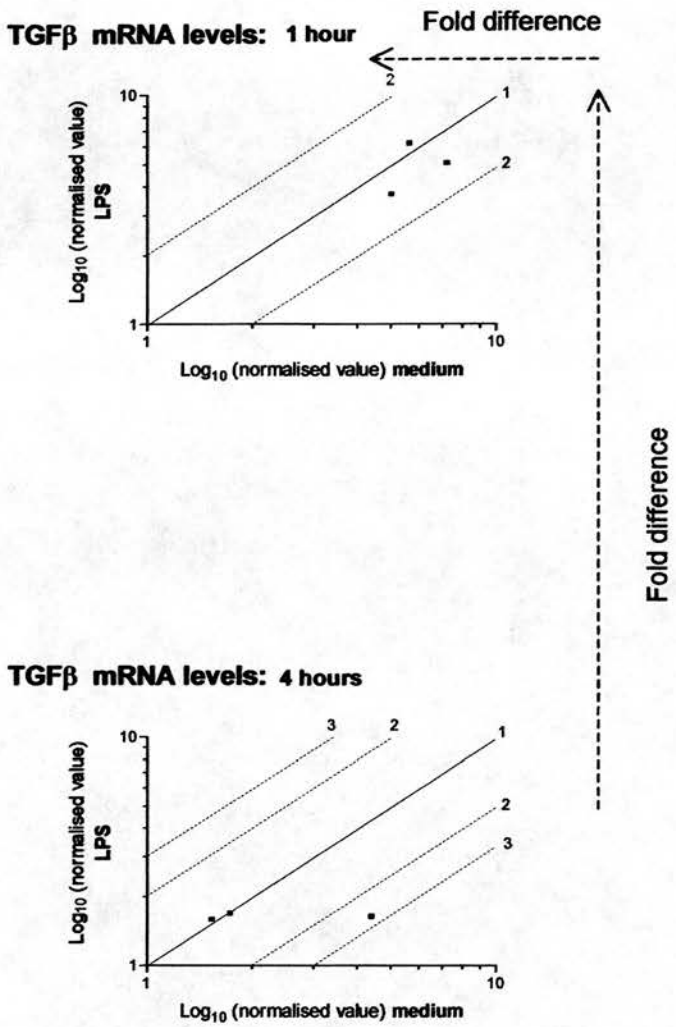

Figure 8a. Expression of TGFβ.

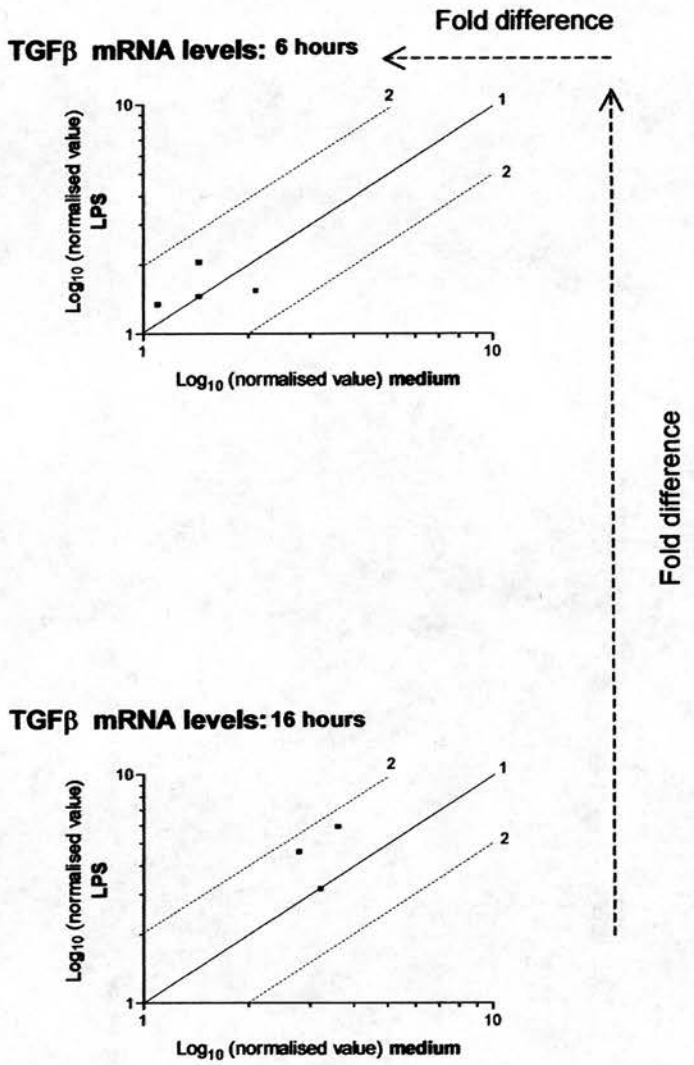

Figure 8b. Expression of TGFβ.

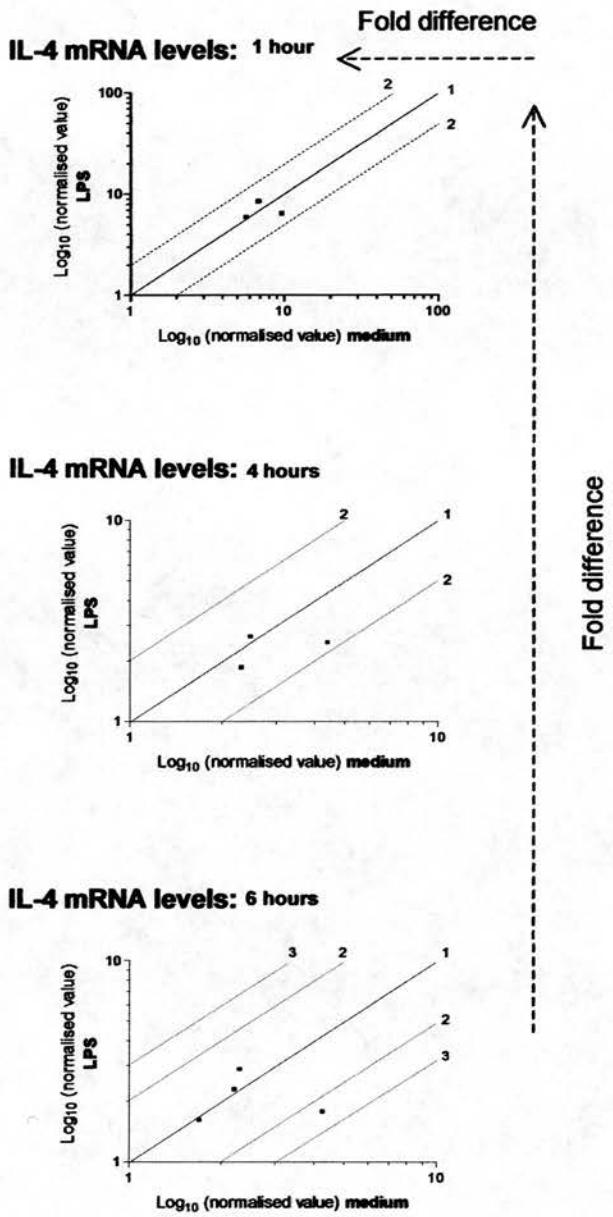

Figure 9a. Expression of IL-4.

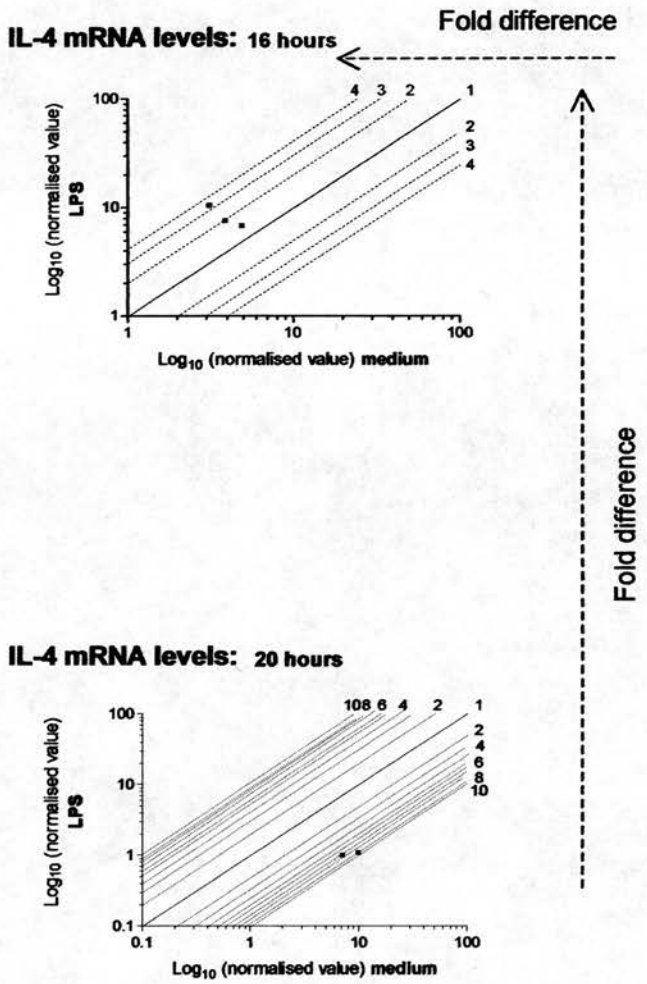

Figure 9b. Expression of IL-4.

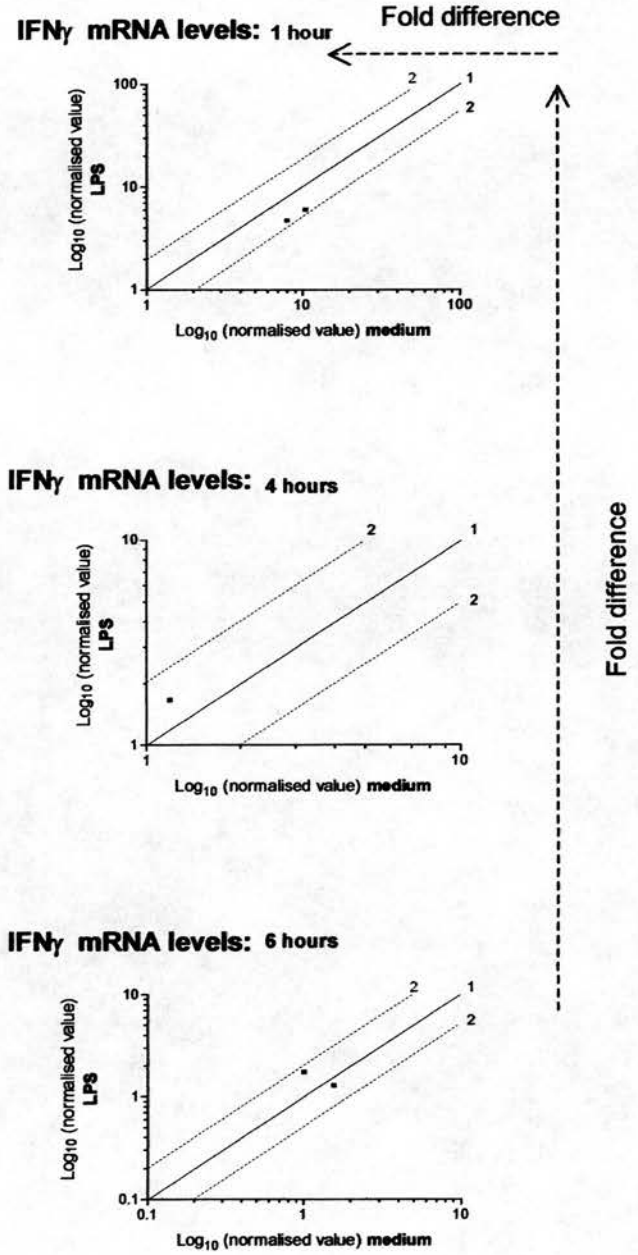

Figure 10a. Expression of IFNγ.

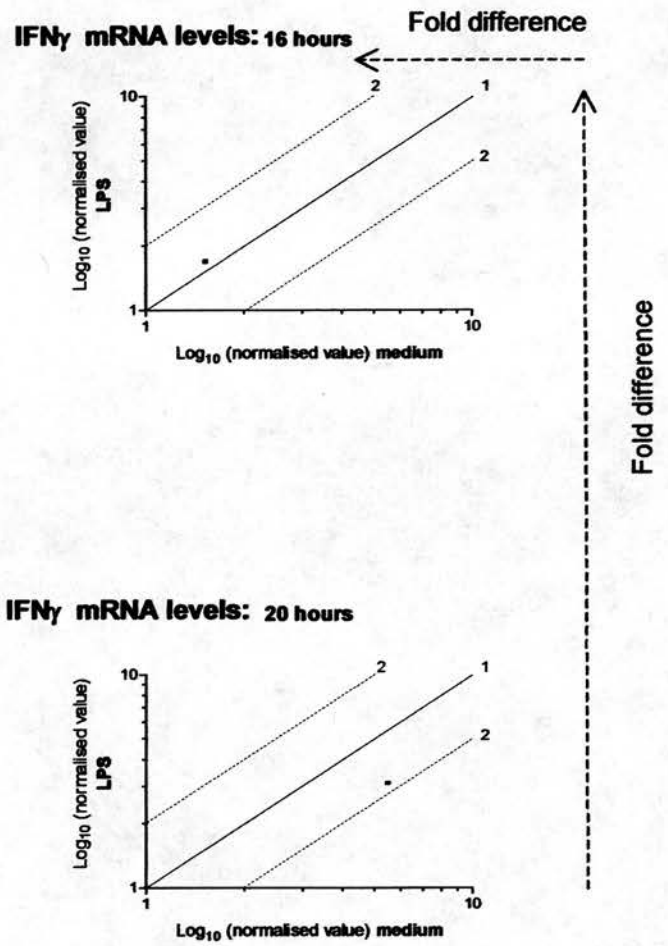

Figure 10b. Expression of IFNγ.

Appendix 7b. Ratio of cytokine mRNA levels in alveolar macrophages enriched by adherence; LPS stimulated versus medium controls.

The RPA was used to evaluate cytokine mRNA abundance in sheep adherent alveolar macrophages cultured with or without 1 μg/ml LPS. The levels of individual transcripts were normalised relative to GAPDH expression, and expressed as an arbitrary unit. The ratio of expression of each cytokine in LPS stimulated cultures is plotted relative to medium controls on a $\log_{10}$ scale, individual samples are plotted as a single point. Where no transcripts were detected in either the control or LPS stimulated cells, a value of zero is given, therefore comparisons of the $\log_{10}$ value of these data cannot be made, and are not included in these graphs. Fold differences of cytokine expression between experimental groups are indicated on the graphs. These experiments involved cells isolated from three sheep. The figures are as follows:

1 IL-12 p40: 1, 4, 12 hours

2 IL-10: 1, 4, 12 hours

3 IL-6: 1, 4, 12 hours

4 GM-CSF: 1, 4, 12 hours

5 IL-8: 1, 4, 12 hours

6 IL-1β: 1, 4, 12 hours

7 TNFα: 1, 4, 12 hours

8 TGFβ: 1, 4 hours

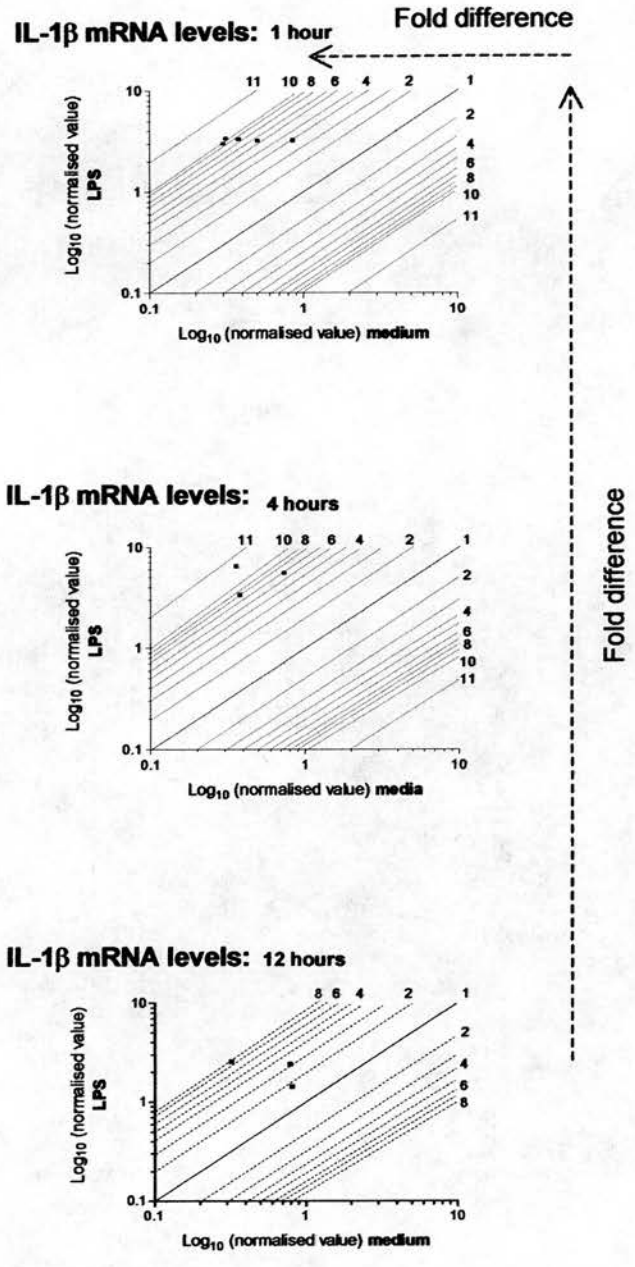

Figure 1 Expression of IL-1β.

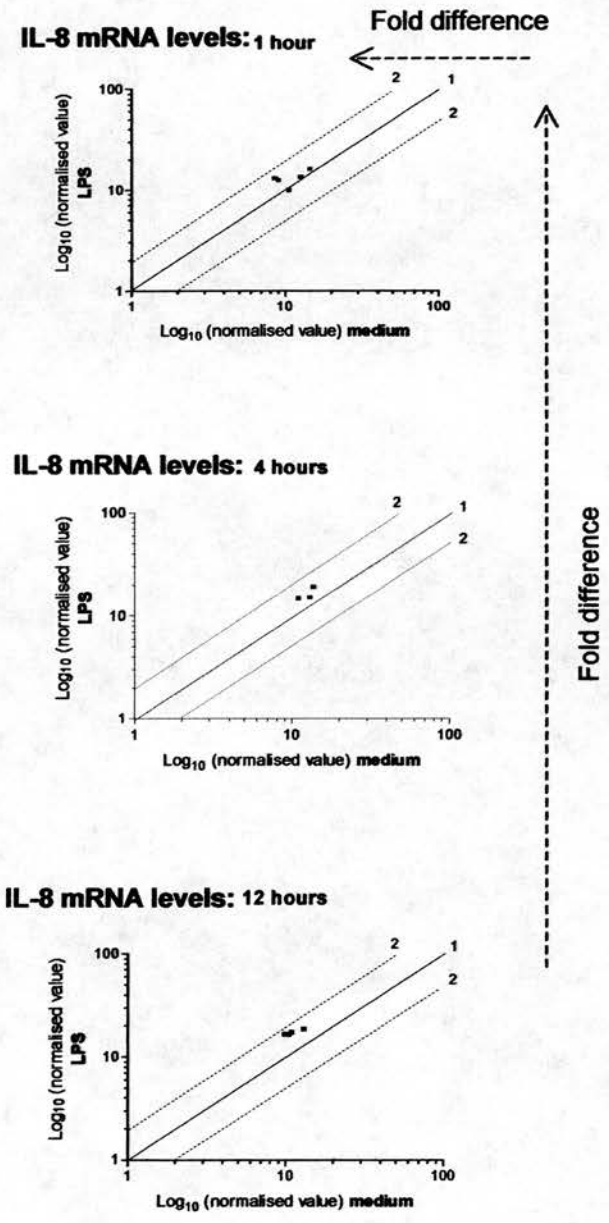

Figure 2 Expression of IL-8.

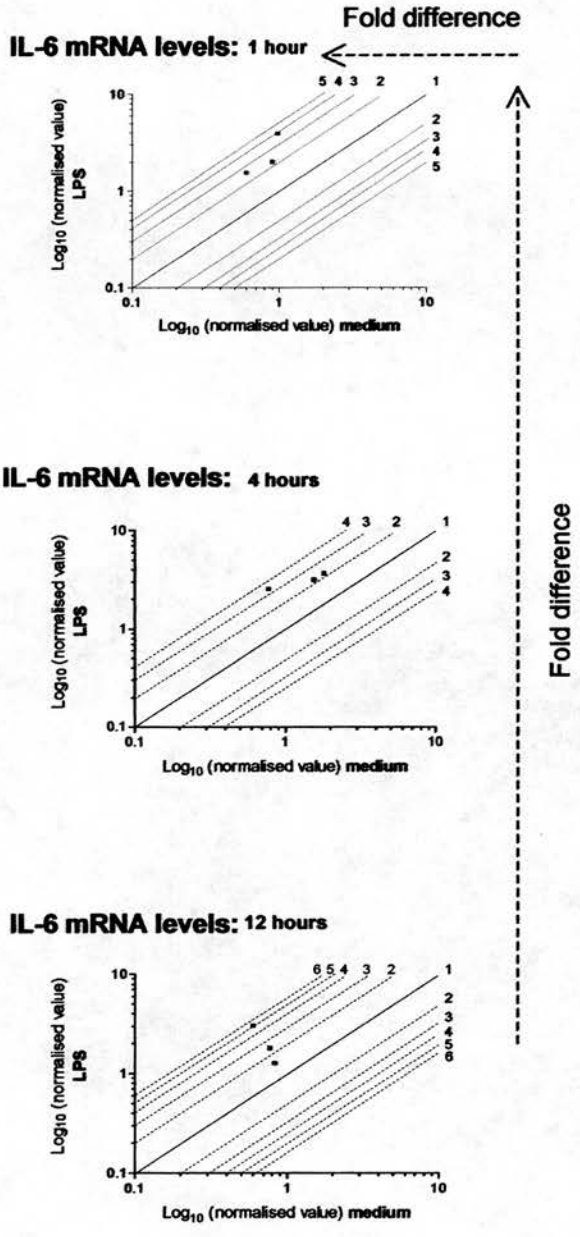

Figure 3 Expression of IL-6.

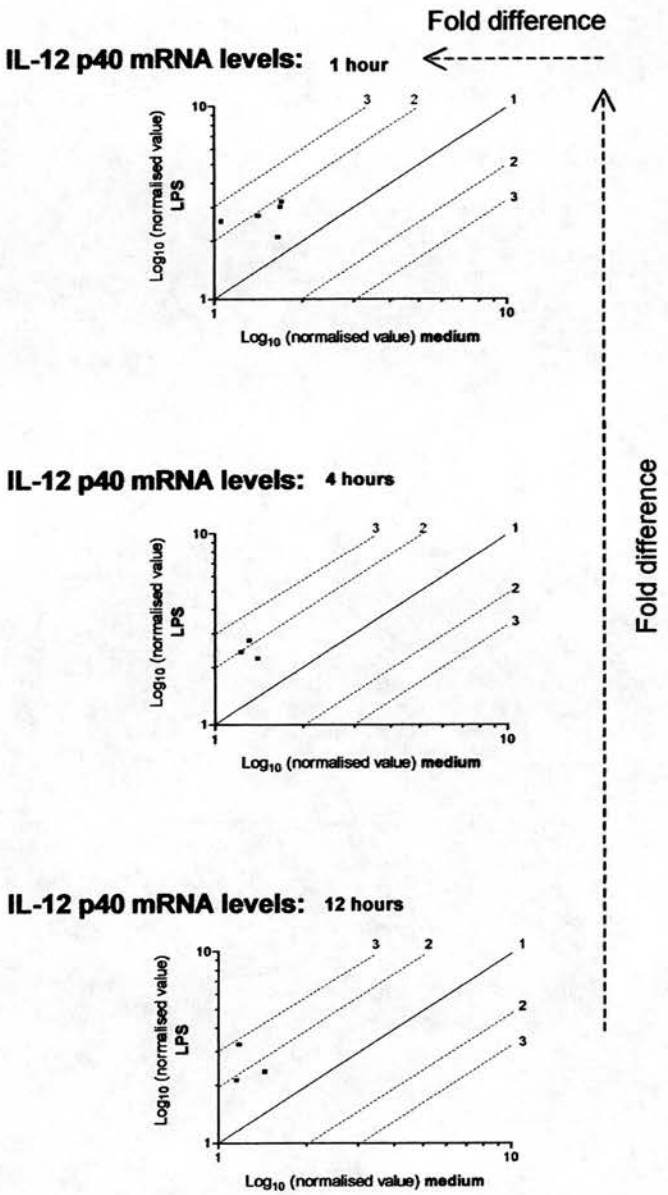

Figure 4 Expression of IL-12 p40.

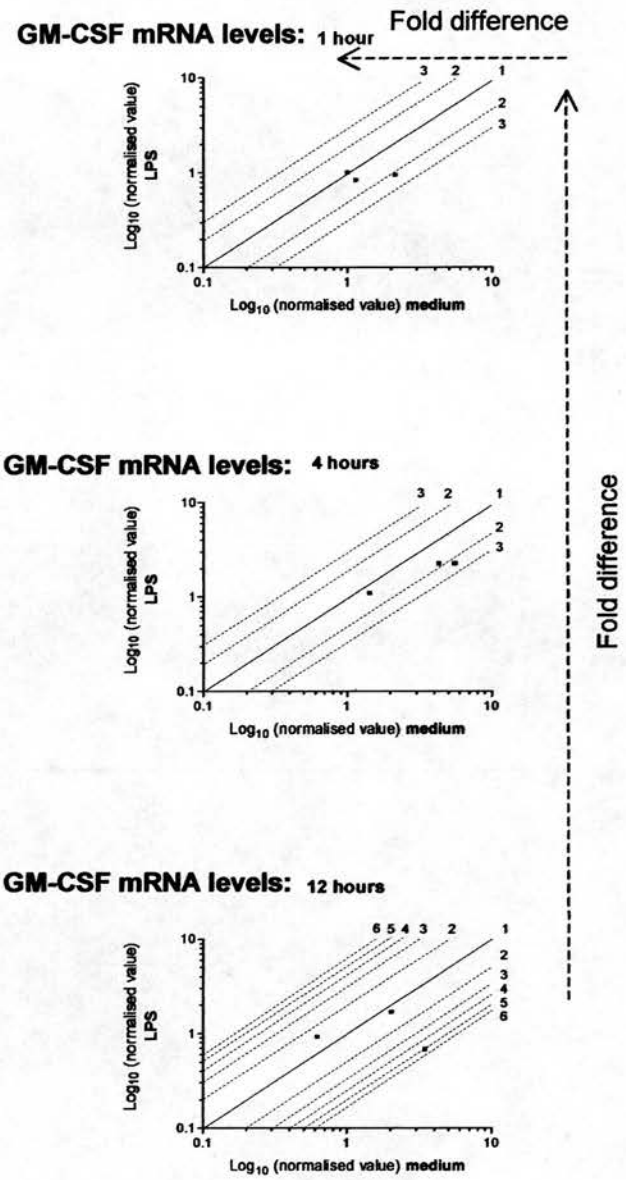

Figure 5 Expression of GM-CSF.

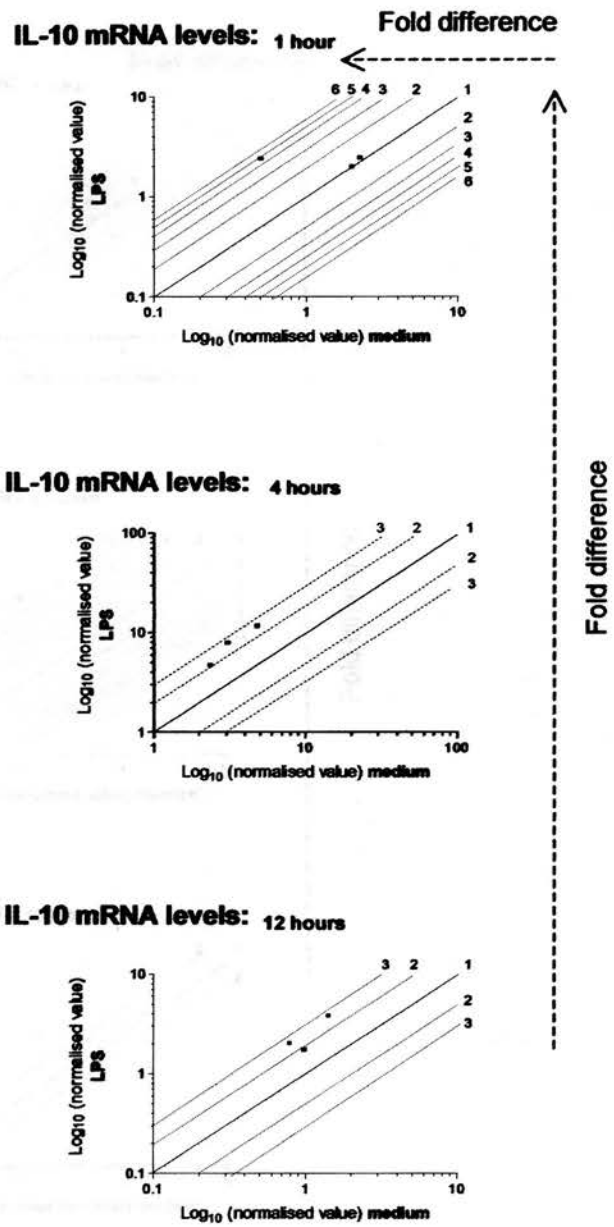

Figure 6 Expression of IL-10.

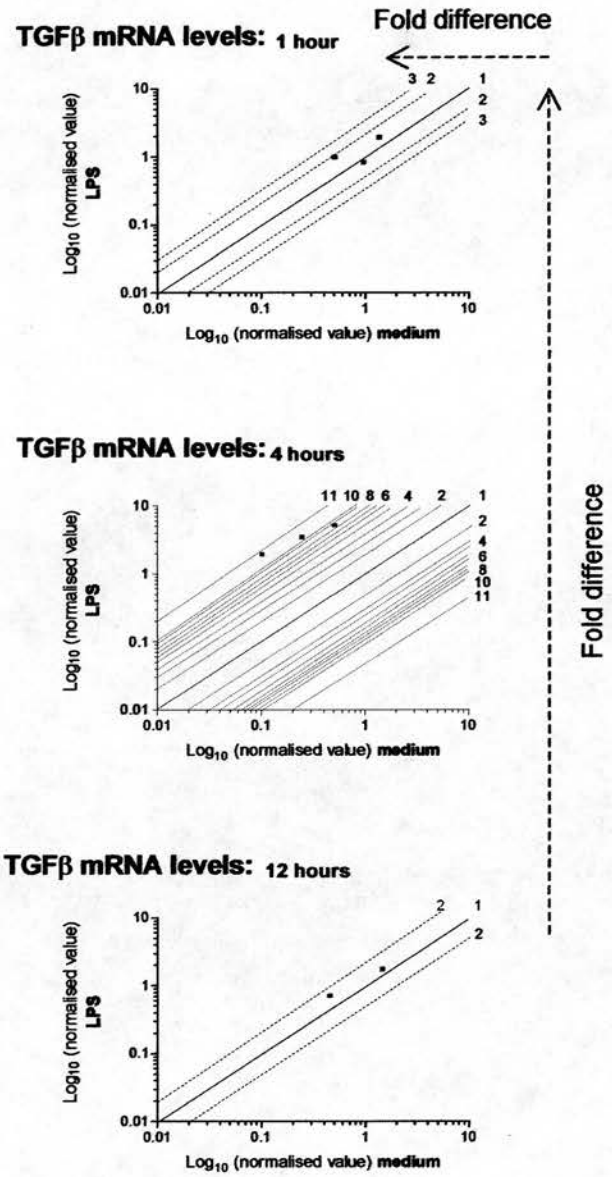

Figure 7 Expression of TGFβ.

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Patterns of cytokine gene expression of naïve and memory T lymphocytes in vivo

Anton G. Gossner[a], Samantha Bailey[a], Nora Hunter[b], John Hopkins[a,*]

[a]*Laboratory for Clinical and Molecular Virology, The University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK*
[b]*IAH Neuropathogenesis Unit, King's Buildings, West Mains Road, Edinburgh, UK*

Abstract

Large-scale lymphocyte recirculation occurs only at the level of secondary lymphoid tissue. Cells enter lymph nodes via afferent lymph from the tissue and via arterioles from the blood. They exit only via the efferent duct. Afferent and efferent lymphocytes have distinct phenotypes; afferent lymphocytes have a 'memory' phenotype, being $CD62L^-/CD45RA^-$ and expressing high levels of CD2 and CD11a; efferent cells are largely 'naïve', being $CD62L^+/CD45RA^+$ with low levels of CD2 and CD11a. We will show that functionally the efferent lymphocytes, like cells from the blood and spleen, can be activated in vitro only by dendritic cells. However, afferent lymphocytes are less stringent in their activation requirements and can be stimulated by both macrophages and dendritic cells. To explain these functional differences we have developed a multiprobe RNAase protection assay for 13 sheep cytokines (IL-1β, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, GMCSF, IFNγ, TGFβ and TNFα) and two housekeeping genes (ATPase and GADPH). We have used this assay to measure the constitutive expression of cytokine mRNA in MACS-purified CD4+ and CD8+ T lymphocytes from both lymphoid compartments.

Keywords: Cytokines; T lymphocytes; Memory figure

1. Introduction

The physiological processes of the immune system, especially adaptive immune responses, are dynamic processes dependent upon the co-ordinated interaction of antigen-trapping accessory cells and lymphocytes in specialised peripheral lymphoid organs such as lymph nodes. The patterns of lymphocyte recirculation through different immunological compartments have been well defined in the sheep. Blast cells from peripheral nodes home to the spleen and lymph nodes, and within this small recirculating lymphocyte pool there are subsets which preferentially recirculate thorough the skin, peripheral nodes or mucosal associated lymphoid tissue (Mackay et al., 1988; Abernethy et al., 1991). The afferent lymph selectively conveys T lymphocytes, a small proportion of B lymphocytes, afferent lymph dendritic cells (ALDCs) and macrophages (Hall and Morris, 1965; Smith et al., 1970). The ALDCs are professional APCs involved in the carriage of antigen in an immunogenic form from the skin to the draining lymph node.

Efferent lymph contains only lymphocytes that enter the lymph node, either from the blood or the afferent lymphatics of the node and leave via the efferent, lymphatics within the lymph plasma, ultimately back into the blood (Hall and Morris, 1962; Smith et al., 1970). The lymph node is a central focus of the immune

* Corresponding author. Tel.: +44-131-650-6169; fax: +44-131-650-6511.
E-mail address: john.hopkins@ed.ac.uk (J. Hopkins).

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system and the use of cannulation of the afferent and efferent lymphatics provides a unique approach to the in vivo analysis of immune cell populations within the different immunological microenvironments of single lymph nodes in the sheep. There are considerable phenotypic and functional differences between the cells in blood and afferent lymph suggesting that there is selectivity in the extravasation of cells from the blood across the endothelium into the peripheral tissues. Emigration of afferent and efferent T lymphocytes, with their distinct phenotypes and function, can be demonstrated by the circulation of naïve and memory T lymphocytes within an individual animal (Mackay et al., 1996).

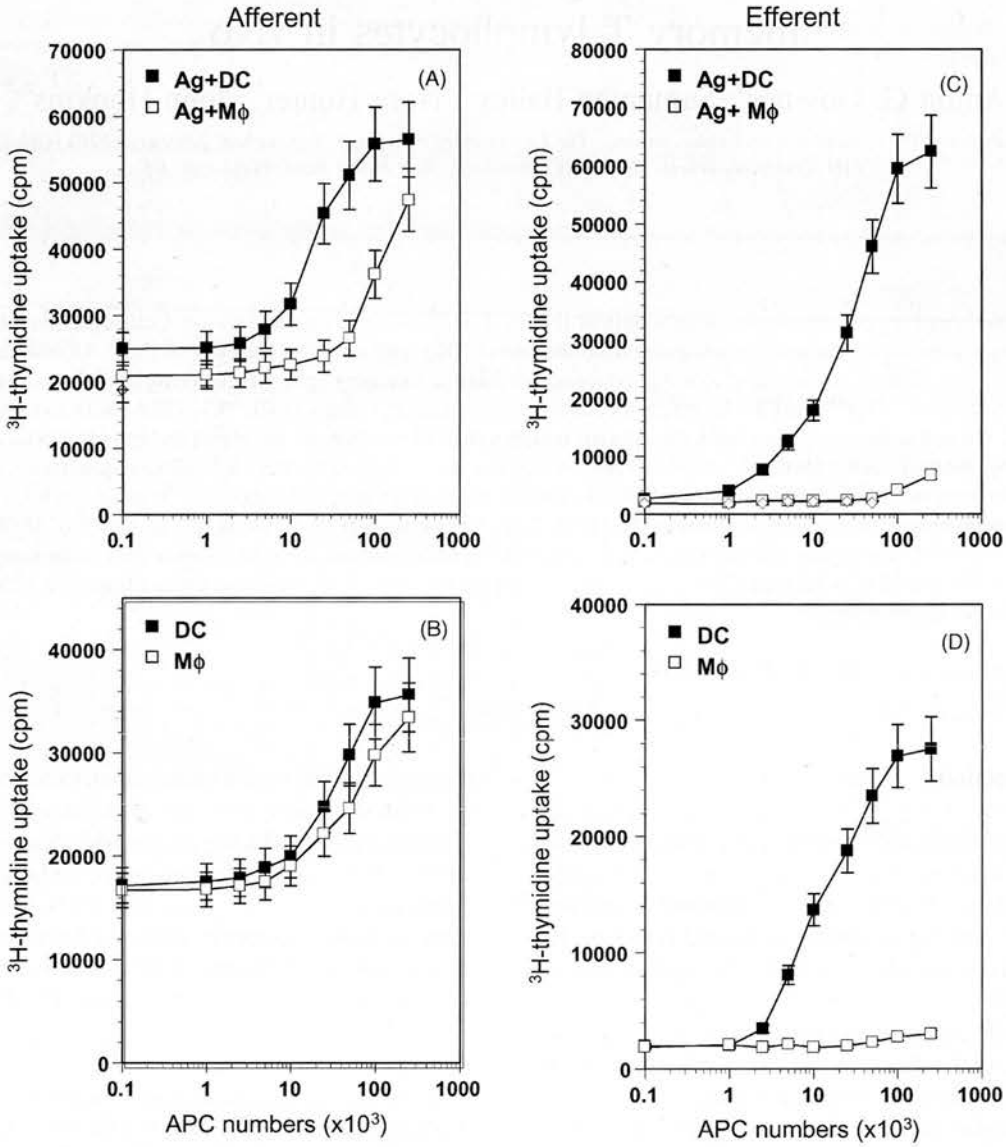

Fig. 1. Proliferation of CD4+ lymphocytes from both afferent and efferent lymph in vitro. In vitro proliferation assays of CD4+ T cells from afferent (A and B) or efferent (C and D) lymph stimulated with 25 μg/ml ovalbumin (A and C) or allogeneic APC (B and D). 5×10^4 lymphocytes were cultured with irradiated afferent DC (DC) or mammary macrophages (Mϕ) for 5 days and then labelled with ^{3}H-thymidine for 5 h.

The blood contains T lymphocytes of both naïve and memory phenotypes though afferent lymph T lymphocytes are mainly of the memory cell phenotype and efferent T lymphocytes draining the lymph node are mainly of a naïve phenotype. Mature lymphocytes, both CD4+ and CD8+, recirculate between the blood and the tissues via the lymph nodes. Immunological memory is disseminated from the node via the efferent lymph, where exit of T lymphocytes following antigen stimulation is non-random despite the fact that cells from the efferent lymph have a 'naïve' phenotype.

A total of 90% of the lymphocytes within a lymph node are derived from the blood, the remainder are from lymphocyte proliferation within the node and afferent lymph (Hall and Morris, 1965). CD4+ cells are selectively enriched over CD8+ T lymphocytes in lymph nodes.

To investigate the relationships between functional activities of lymphocytes and their migratory properties, we have developed a multiprobe RNAase protection assay (RPA) for 13 sheep cytokines (IL-1β, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, GMCSF, IFNγ, TGFβ and TNFα), as well as two housekeeping genes (ATPase and GADPH). Cannulation of the prefemoral "pseudo-afferent" and efferent lymphatics of sheep allows the isolation of 'resting' trafficking lymphoid cells to be analysed for their constitutive cytokine mRNA expression.

2. Naïve, memory and effector lymphocytes

Afferent lymph contains ALDCs, T lymphocytes and B lymphocytes, all migrating from the tissues to the local lymph node. The afferent T lymphocytes express an activated phenotype ($CD2^{HI}$ $CD58^{HI}$ $CD44^{HI}$ $CD11a^{HI}$, high level expression of MHC II DQ^{+} and CD25) and high levels of adhesion molecules but are $CD45RA^{-}$ compared to efferent lymph cells (Mackay et al., 1990, 1992; Abitorabi et al., 1996). In terms of function they respond to soluble antigen (Fig. 1A) and alloantigen presented by both ALDCs and macrophages (Fig. 1B).

Efferent lymph contains >99% lymphocytes with a high proportion of CD4+ T lymphocytes and B lymphocytes compared to CD8+ and γδ T lymphocytes. T lymphocytes isolated from efferent lymph ($CD2^{LO}$ $CD58^{LO}$ $CD44^{LO}$ $CD11a^{LO}$, class II MHC DQ^{-} and $CD25^{-}$) are $CD45RA^{+}$ and express low levels of adhesion molecules (Mackay et al., 1990, 1992; Abitorabi et al., 1996). These T lymphocytes are highly responsive to both soluble antigen and alloantigen when presented by ALDCs. They respond very weakly to soluble antigen presented by macrophages and are non-responsive to allogenic macrophages in a mixed leukocyte reaction (Fig. 1D).

Using MACS-purified CD4+ and CD8+ T lymphocytes from afferent and efferent lymph, the constitutive expression of cytokine mRNA in both cell populations using a multiprobe RPA was analysed. Preliminary results identified T lymphocytes from "resting" afferent lymph that expressed mRNA for the cytokines IL-1β, IL-3, IL-8, IFNγ, TNFα and TGFβ using this assay system (Fig. 2). With T lymphocytes

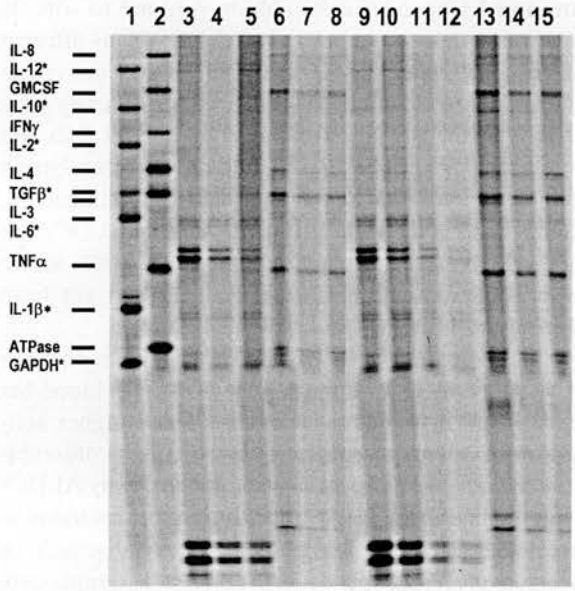

Fig. 2. Measurement of cytokine mRNA levels using an RNAase protection assay. The RNAase protection assay is a sensitive and specific method for the quantitation of mRNA species. The probe templates were generated by RNA polymerase directed synthesis of ^{32}P-labelled anti-sense RNA from a cDNA template. The purified-labelled probes were hybridised with the target RNA isolated from both afferent (lanes 9–15) and efferent (lanes 3–8) lymph cells. After hybridisation was completed, ribonucleases specific for ssRNA digested unhybridised RNA and probe. The "RNAase-protected" probes and undigested probe markers (lanes 1 and 2) were resolved on a denaturing PAGE and were visualised by phosphor imaging. Probe set 1* was used with the samples in lanes 3–5 and 9–12. Probe set 2 was used with the samples in lanes 6–8 and 13–15.

from "resting" efferent lymph expressed mRNA for the cytokines IL-2, IL-3, IFNγ, and TNFα were detected (Fig. 2).

3. Discussion

'Resting' efferent lymph T lymphocytes with their naïve phenotype require activation by ALDCs to respond to soluble antigen, yet 'resting' afferent lymph T lymphocytes do not require ALDCs for activation, as they are activated by macrophages, though they still also respond to ALDCs. This clearly demonstrates that afferent lymph responder cells have 'immunological memory', thus enabling them to respond to antigen presentation by macrophages. Conversely with 'resting' efferent lymph, there are few unprimed responder cells able to respond to soluble antigen without stimulation by ALDCs. Thus afferent lymph T lymphocytes demonstrate a lower activation threshold, i.e. 'memory', which is functionally illustrated by their ability to respond both to ALDCs and mammary macrophages. In contrast efferent lymph T lymphocytes have a higher activation threshold, confirmed by their ability to respond to ALDCs but not macrophages. Both sets of lymphocytes act in response to stimulation from ALDCs and yet have distinctly different phenotypes.

The responder cells of efferent lymph with their naïve phenotype have recirculated from the blood but have no 'immunological memory' and a higher activation threshold, which is only overcome by presentation of both soluble antigen and alloantigen by ALDCs and not macrophages. 'Naïve' T lymphocytes travel to areas of secondary lymphoid organs in search of antigen presented by dendritic cells. T lymphocyte memory is demonstrated by afferent lymph cells ability to proliferate in response to soluble antigen presentation by macrophages but the presence of memory effector cells is demonstrated by their ability to respond to alloantigen presentation by both ALDCs and macrophages though to a lesser extent than with soluble antigen.

Further work needs to be done to map and define naïve, memory and effector cells before the issue of naïve and memory phenotype and distribution can be resolved. These new RPA multiprobe sets provides valuable tools for the simultaneous quantitative determination of gene expression of multiple ovine cytokines of both constitutive and inducible type.

Acknowledgements

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